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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Andrew M. Scharenberg
Serial No.:	09/869,486
Confirmation No.:	4102
Filed:	January 4, 2002
For:	CHARACTERIZATION OF THE SOC/CRAC CALCIUM CHANNEL PROTEIN FAMILY
Examiner:	Olga N. Chernyshev
Art Unit:	1646

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. 1.132

Sir:

I, Jean-Pierre Kinet, declare that:

Background and Experience

1. I am a consultant and a member of the Scientific Advisory Board at Synta Pharmaceuticals Corp., the exclusive licensee of the above-identified application ("Exclusive Licensee").

2. I have 23 years of experience in the area of molecular and cellular biology of the immune system, specifically ions channels in cells of the immune system. My Curriculum Vitae is attached as Exhibit A.

3. I am currently a Director of the Division of Allergy and Immunology in the Department of Pathology at Beth Israel Deaconess Medical Center in Boston, Massachusetts ("Beth Israel"), a position I have held for the past five years. I am a Lecturer on molecular and cellular biology at Harvard University, a position I have held for the past ten years. I am also an ad-hoc member of the Allergy and Immunology program Advisory Subcommittee of the National Advisory Allergy and Infectious Diseases Council at the National Institutes of Health, a

position I have held for the past six years.

4. I have received nine United States patents (U.S. Patent Nos. 5,639,660; 5,770,396; 5,807,998; 5,965,605; 6,165,744; 6,165,799; 6,171,803; 6,423,501; 6,602,983) in the areas of immunoglobulin receptors and monoclonal antibodies, and I am an inventor on additional pending United States patent applications in the areas of: methods for screening compounds capable of depleting mast cells, novel components of cell signaling cascades, use of potent, selective and non-toxic c-kit inhibitors for treating bacterial infections, use of tyrosine kinase inhibitors for treating allergic diseases, and novel calcium channels that are differentially expressed in non-excitable cells.

5. Dr. Andrew Scharenberg, the sole named inventor in the above-identified United States patent application, was a colleague of mine at Beth Israel and I am familiar with the invention described and claimed in this patent application. I have thoroughly read and am familiar with the patent application and I have read and am familiar with the outstanding Office Action in this case, mailed on December 23, 2004. In addition, I was present at an interview on April 5, 2005 with Primary Examiner Olga N. Chernyshev, Supervisory Examiner Anthony Caputa, John Van Amsterdam, attorney for Applicant, and Wendy Rieder, attorney for Exclusive Licensee, in which the outstanding Office Action was discussed.

State of the Art at the Time the Application was Filed

6. Below I have summarized several articles and a patent that in my opinion represent the state of the art at the time the above-referenced patent application was filed. The articles and patent, all of which were published prior to the filing of the above-identified application, demonstrate that small molecule modulators of widely expressed proteins (ion channels and calcineurin phosphatase) could be identified, and that these molecules were useful as immunosuppressants.

7. Cahalan, M.D. and Chandy, K.G., "Ion Channels in the Immune System as

Targets for Immunosuppression," *Current Opinion in Biotechnology* (1997), *8*:749-758 (Exhibit B)

(i) Activation of T cells. Exhibit B summarizes the cascade of events that lead to the activation of T cells in response to antigen receptor engagement. When a T cell binds to an antigen, tyrosine kinases are activated that lead to the generation of inositol 1,4,5-triphosphate (IP_3). IP_3 causes the release of stored cellular Ca^{2+}, in addition to the influx of extracellular Ca^{2+} causing the cytoplasmic Ca^{2+} concentration to rise. The rise in cytoplasmic Ca^{2+} concentration activates the phosphatase calcineurin which dephosphorylates NF-AT, a nuclear transcription factor which is located in the cytoplasm. Once NF-AT is dephosphorylated, it can move into the nucleus where it binds to the promoter sequence for interleukin 2 (IL-2) and begins to transcribe the gene. IL-2 is a T cell mitogen and leads to T cell proliferation. (Exhibit B, page 749, Col. 1-2, paragraph 1 of the Introduction section and Figure 1).

(ii) Approaches to inhibiting T cell activation. Cyclosporin A and FK506 are two agents that had been used for many years to treat patients in need of immunosuppression (e.g., patients who had undergone organ transplantation) These drugs inhibit the dephosphorylation activity of calcineurin and thereby suppress immune response by suppressing the transcription of IL-2. Exhibit B states that ion channels that control the influx of Ca^{2+} into T cells may also be an effective target for pharmacological modulation of the immune response since a rise in Ca^{2+} is necessary to activate calcineurin. (Exhibit B, the paragraph spanning page 749, Col. 2 to page 750, Col. 1).

Exhibit B also summarizes the activity of a voltage gated K^+ ion channel, Kv1.3, which is involved in determining the driving force of Ca^{2+} entry into T cells. Kv1.3 opens in response to membrane depolarization that occurs when Ca^{2+} enters the cell and allows K^+ to pass out of T cells to maintain the resting membrane potential of -50 to -60 mV. (Exhibit B, page 750, Col. 1, 2nd full paragraph). Thus, a positive driving force for Ca^{2+} entry into the cell is maintained. Margatoxin, a selective inhibitor of Kv1.3, was found to be a safe and significantly more effective immunosuppressant than FK506 *in vivo*. (Exhibit B, page 750, Col. 2, 2nd full paragraph).

8. Baker, et al., "Triterpene Derivatives with Immunosuppressant Activity," U.S. Patent No. 5,679,705, published on October 21, 1997 (Exhibit C)

Kv1.3 modulators inhibit T cell activation. Exhibit C discloses triterpene derivatives that are immunosuppressive agents that are useful in treating autoimmune diseases and preventing rejection of organ transplants (Exhibit C, Col. 2, lines 35-54). Exhibit C states that the compounds disclosed block Kv1.3 and inhibit T cell activation (Exhibit C, Col. 37, lines 20-22). Kv1.3 is a voltage gated potassium channel that is found in neurons, blood cells, osteoclasts and T-lymphocytes (Exhibit C, Col. 36, line 66 to Col. 37, line 1).

9. Jensen, *et al.*, "Characterization of the Cloned Human Intermediate-Conductance Ca^{2+}-activated K^+ Channel," *Am. J. Physiol.* (Sept. 1998), *275*:C848-C856 (Exhibit D)

IK modulators inhibit T cell activation. Exhibit D describes the tissue distribution of human intermediate-conductance, Ca^{2+}-activated K^+ channels (hIK). These channels are present in salivary gland, placenta, lung, trachea, liver, colon, thymus, kidney, bone marrow tissue, and in T and B lymphocytes (Exhibit D, page C854, Col. 1, 1st paragraph). hIK channels are upregulated in T and B cells upon activation with antibodies or phorbol 12-myristate 13-acetate, suggesting that they have an important function in immune system activation (Exhibit D, page C855, Col. 1, the last 6 lines of paragraph 1).

10. Jensen, *et al.*, "Inhibition of T Cell Proliferation by Selective Block of Ca^{2+}-activated K^+ channels," *Proc. Natl. Acad. Sci. USA* (Sept. 1999), *96*:10917-10921 (Exhibit E)

(i) IK channels are present in a number of tissue types. Exhibit E indicates that IK channels are K^+ ion channels which are activated by a rise in cytoplasmic Ca^{2+} concentration. IK channels are found in a number of tissues, such as spleen, thymus, endothelial cells, epithelial

cells, peripheral blood leukocytes and T cells (Exhibit E, page 10917, Col. 1, 1st paragraph after abstract). In T lymphocytes, they open after initial antigen stimulation of the T cell receptor hyperpolarizes the membrane and increases the influx of Ca^{2+} into the cell. IK channels release K^+ from the cell to maintain membrane hyperpolarization and maintain an inwardly directed driving force for influx of Ca^{2+} (Exhibit E, page 10917, paragraph spanning Col. 1 and Col. 2).

(ii) IK channel modulators inhibit T cell proliferation. Exhibit E demonstrates that inhibition of IK by nitrendipine, clotrimazole and charybdotoxin leads to inhibition of the proliferative T cell response and inhibition of the release of IFN-γ from active T cells. Blocking IK channels interferes with the rise in intracellular Ca^{2+} which is recognized as an obligatory step in the cascade of signals that result in T cell proliferation. (Exhibit E, page 10920, 1st paragraph of the Discussion section).

11. Klee, C.B., *et al.*, "Regulation of the Calmodulin-stimulated Protein Phosphatase, Calcineurin," *The Journal of Biological Chemistry* (May 29, 1998), *273*(22), 13367-13370 (Exhibit F)

(i) Calcineurin is present in a number of tissue types. Exhibit F discloses that calcineurin is a protein phosphatase that is broadly distributed but is especially abundant in neural tissues. Calcineurin is particularly abundant in the striatal and hippocampal neurons. Hippocampal neuron activation of calcineurin results in inhibition of the release of the neurotransmitters glutamate and γ-aminobutyric acid, and desensitizes the postsynaptic NMDA receptor-coupled Ca^{2+} channels. Calcineurin is thought to play a role in long term potentiation, depression and long term memory (Exhibit F, page 13369, 4th full paragraph).

(ii) Calcineuerin is a well accepted immunosuppressive target. The function of calcineurin was difficult to identify until it was discovered that it was the target of the immunosuppressive drugs FK506 and cyclosporin A, and it played an essential role in T cell activation (Exhibit F, page 13367, Col. 1, paragraph 1). Calcineurin binds to the transcription factor NF-AT and dephosphorylates it allowing NF-AT to translocate to the nucleus where it

binds to the promoter sequence for IL-2, a T cell growth factor (Exhibit F, page 13369, Col. 2, 2nd full paragraph).

Teachings from the Literature at the Time of Filing

12. Increase in Ca^{2+} concentration is vital for lymphocyte proliferation. At the time the above-identified application was filed, the references described above show that it was known that an increase in Ca^{2+} concentration is vital for lymphocyte proliferation. The rise in cytoplasmic Ca^{2+} concentration was known to activate calcineurin, causing it to dephosphorylate NF-AT which in turn allows NF-AT to translocate into the nucleus. Once in the nucleus, NF-AT stimulates IL-2 transcription by binding to the promoter sequence for IL-2, a T cell mitogen that stimulates T cell proliferation.

13. Inhibiting T cell proliferation by modulating targets expressed in other tissues was an accepted approach at the time this application was filed. At the time the above-identified application was filed, the references described above show that the mechanism of action of the small molecule immunosuppressive drugs cyclosporin A and FK506 was inhibition of calcineurin, which resulted in inhibition of IL-2 transcription. Despite the fact that calcineurin has a broad tissue distribution and is particularly abundant in neural tissue, cyclosporin A and FK506 had been successfully used in humans for many years as immunosuppressants.

At least two ion channels, Kv1.3 and IK, were also known at the time the above-identified application was filed to be involved in maintaining a positive driving force for Ca^{2+} entry into lymphocytes by maintaining the cell membrane hyperpolarization. A positive driving force for Ca^{2+} entry into lymphocytes is necessary for calcineurin activation. Both Kv1.3 and IK were known to be expressed in lymphocytes, such as T cells and B cells, but were also known to have widespread expression in other tissues. However, at the time that this patent application was filed, potent, selective blockers of Kv1.3 were known and had been found to inhibit immune responses *in vivo* without serious toxicity. In addition, at the time the instant invention was made, blockers of IK had been shown to inhibit T cell proliferation.

14. Applicant made reasonable predictions concerning the utility of SOC3/CRAC2. Since it was known that regulation of Ca^{2+} signaling pathways in T lymphocytes could be used to control lymphocyte proliferation, I believe that it was reasonable for Applicant to predict that the SOC3/CRAC2 channel, which gates Ca^{2+} entry into a cell, would be involved in lymphocyte proliferation. Moreover, calcineurin, Kv1.3 and IK, which are all expressed in tissues and cell types other than lymphocytes, had been successfully targeted by immunosuppressant compounds, some of which were used to treat humans with autoimmune disorders and to prevent organ transplant rejection. Therefore, I also believe that it was reasonable for Applicant to expect that, although the SOC3/CRAC2 channel was expressed in tissues and cell types other than lymphocytes, the channel could be used to identify useful small molecule inhibitors of SOC3/CRAC2 that inhibit lymphocyte proliferation (i.e., immunosuppressive agents).

15. Proof of Concept. After the filing date of the instant application, Exclusive Licensee of the instant patent application definitively identified a series of small molecule compounds that modulate the activity of the SOC3/CRAC2 channel. These compounds were identified using the SOC3/CRAC2 channel disclosed and claimed in the instant application to screen for modulators. Two of the compounds have been shown to be effective *in vivo* in a murine mixed lymphocyte reaction (MLR), which is an accepted model for organ transplant rejection. In addition, the compounds were shown to have no acute toxicity in animal studies and no animal deaths occurred at high doses of the compound (100 mg/kg) (See Declaration under 37 C.F.R. § 1.132 by Dr. Michael Xie, filed herewith). In my opinion, these results support the reasonable predictions made by Applicant concerning the utility of the SOC3/CRAC2 channel.

Professional Opinion

16. Based on the foregoing, it is my professional opinion that, at the time the above-referenced patent application was filed, Applicant had a reasonable expectation that the SOC3/CRAC2 channel could be used to successfully identify small molecule inhibitors that inhibit lymphocyte proliferation (e.g., immunosuppressive agents), even though the channel was

expressed in tissue types and cells other than lymphocytes.

17. In addition, based on the foregoing it is also my opinion that, at the time the above-referenced patent application was filed, it was well known in the art that agents blocking lymphocyte proliferation are effective immunosuppressive agents that can be used against certain disorders, such as preventing organ transplant rejection and treating autoimmune disease. It was reasonable for Applicant to expect as of the date this application was filed that modulators of the SOC3/CRAC2 channel would have real-world use as immunosuppressive agents.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued therefrom.

04.20.05
Date

Jean-Pierre Kinet, M.D.

Jean-Pierre Kinet

Curriculum Vitae

Office Address: Beth Israel Deaconess Medical Center
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Home Address: 3 Hunt Road
Lexington MA 02421
Tel: 781-674-1446

Date and Place of Birth: October 23, 1953 - Vielsalm, Belgium

Citizenship: Belgian and French

Visa Status: Permanent Resident of theUnited States

Marital Status: Married; 5 children

Education:

1974	Bachelor in Medicine, Cum Laude, University of Namur, Belgium
1978	M.D., Magna Cum Laude, University of Liège, Belgium

Pre- and Postdoctoral Training:

1974-1976	Research Fellow, Department of Blood Transfusion, University of Liège, Belgium
1978-1982	Residency in Internal Medicine, University of Liège, Belgium
1982-1987	Visiting Associate, Section on Chemical Immunology, Laboratory of Dr. H. Metzger, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH

Academic Appointments:

1987-1989	Senior Investigator, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH
1989-1995	Head, Molecular Allergy and Immunology Section, National Institute of Allergy and Infectious Diseases, NIH
1995-present	**Professor of Pathology, Harvard Medical School**

Hospital Appointments:

1982-1987	Medical Staff Fellow, Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculo-skeletal and Skin Diseases, NIH
1995-2000	Chief, Laboratory of Allergy and Immunology, Department of Pathology, Beth Israel Deaconess Medical Center, Boston
2000-present	**Director, Division of Allergy and Immunology, Department of Pathology, Beth Israel Deaconess Medical Center, Boston**

Awards and Honors:

1983 NATO Research Award.
1992 American Association of Immunologist Travel Award
1997 Inventor Award from the National Institutes of Health
1999 Pfizer Visiting Professor in Allergic Diseases and Asthma (Vanderbilt University)
2000 Chevalier de l'Ordre National du Mérite de la République Française
2001 Pfizer Visiting Professor in Allergic Diseases and Asthma (Baylor College of Medicine)
2004 Merit award, National Institutes of Health

Societies:
1988 American Association of Immunologists
1989 American Society for Cell Biology
1989 American Society for Biochemistry and Molecular Biology
1989 Society for Leucocyte Biology
1990 American Academy of Allergy and Clinical Immunology
1995 Collegium Internationale Allergologicum
1995 New York Academy of Sciences
1995 American Association for the Advancement of Science

Editorial Boards:
1989-1995 Associate Editor, Molecular Immunology
1990-1995 Associate Editor, The Journal of Immunology
2001- Editorial Board, Immunological Reviews
2004- Editorial Board, The Journal of Experimental Medicine

Ad-Hoc Editor:
New England Journal Of Medicine, Cell, Nature, Science,
Blood, Proceedings of The National Academy of Sciences, Journal of Biological Chemistry,
Analytical Biochemistry, Biochemistry, European Journal of Immunology, Immunology Today,
Journal of Immunological Methods, American Journal of Pathology, Molecular and Cellular Biology,
Molecular Biology of the Cell, Journal of Cell biology, Journal of Clinical Investigation,
Journal of Allergy and Clinical Immunology

Distinguished Lectureships
Annual Lecture of the Histamine Research Society, New-Orleans, Louisiana, March 1989
Distinguished Immunologist Lecture, University of California - San Francisco, April 23, 1990
State of the Art Presentation, 8th Annual Aspen Allergy Conference, July 26, 1990
Lecture of the Distinguished Investigator Program of the Mayo Clinic, May 20, 1993
Zoltan Ovary Lecture, New York University, April 16, 2001
Ishizaka Lectureship. Salk Institute, La Jolla, California, May 29. 2003
Plenary Lecture, Congress of the European Academy of Allergology and Clinical Immunology, Paris, June 2003
Plenary Lecture, AAI Annual Meeting, Denver, May 2003
Keynote Speaker, The Batsheva de Rothschild Workshop on Mast cells, Israel, February 9, 2005

Meetings and Courses
Chairman, Fc Receptor Workshop and Chairman, Symposium on "IgE Receptors and Mast cell Secretion". AAI-FASEB meeting. Atlanta, Georgia, April 91.
Chairman, Session on Signal transduction by Fc receptors. FASEB Summer Research Conference. Fc Receptors: Genes, signaling and Function. July 1993
Chairman, Session on Early Events in Mast Cell Signal Transduction at the second. International Workshop on Signal Transduction in Mast Cell Activation and Development. Bethesda, Maryland. February 1995.
Co-chairman and Conference Organizer, Conference on the Biology of Fc Receptors, July 1996
Chairman and Conference Organizer, FASEB summer conference on the Biology of ImmunoReceptors, June 1999

Chairman, Session on BCR and Mast Cell Signaling, FASEB Summer Research Conference. Signal Transduction in the Immune System, July 2000

Chairman, AAI Major Symposium at AAI Annual Meeting, Denver, May 2003

Chairman, Session on Immune Receptors, FASEB Summer Research Conference. Signal Transduction in the Immune System, June 2003.

Teaching Experience:

1989-1995	Lecturer, Course on Molecular and Cellular Mechanism of Immunity. Foundation for Advanced Education in the Sciences, Bethesda, Maryland
1995-	Lecturer, Harvard University, Molecular and Cellular Biology Course, (MCB 268)
1996-	Lecturer, Harvard University, Division of Medical Sciences Courses, (Immunology 301, Immunology 204)

Harvard University Committees:

1996-	Member, Committee on Immunology, Harvard Medical School
1997-	Member, Immunology Graduate Committee, Harvard Medical School
1998	Member, Ad-Hoc Committee of evaluators in the search for Professor of Pathology at the Dana Farber Cancer Institute
1999	Member, Ad-Hoc Committee of evaluators for promotion to Professor of Pediatrics at the Center of Blood Research
1999	Member, Ad-Hoc Committee of evaluators for promotion to Professor of Neurology at the Beth Israel Hospital
1999-	Member, Executive Committee on Immunology, Harvard Medical School
1999-2002	Member, Ph.D. Thesis Examination Committee, Harvard Medical School
2001	Member, Ad-Hoc Committee of evaluators for promotion to Professor of Pediatrics at Children's Hospital
2004	Chairman, Ad-Hoc Committee of evaluators for promotion to Professor of Medicine at Brigham and Women's Hospital

National and International Committees

1990-1997	Ad-Hoc Member, Allergy and Immunology Study Section, National Institutes of Health
1991-1998	Chairman, Nomenclature on Fc receptors, Word Health Organization
1997-2002	Member, Allergy and Immunology Study Section, National Institutes of Health
1997-	Member of the Ad-Hoc Scientific Review Committee of the Pasteur Institute, Paris, France
1997-	Ad-Hoc Member of the National Advisory Allergy and Infectious Diseases Council, Allergy and Immunology program Advisory Subcommittee, National Institutes of Health
1998-2001	Block Chairman, Program Committee, American Association of Immunology
2000	Member of the Scientific Review Committee of the Austrian Research Fund
2005-	Member, Scientific Council, "Laboratoire Européen Associé FNRS-INSERM", Institut d'Immunologie Médicale, Université Libre de Bruxelles, Belgium

Board Membership

1995	Scientific Founder of Astarix Institute Inc, Lexington, Massachusetts
1995-2002	Member, Scientific Advisory Board, Heska Corp., Fort Collins, Colorado
1996-1997	Member, Board of Trustees, The French-American International School of Boston
1997-2000	Chairman, Board of Trustees, The French-American International School of Boston
1999-	Member, Scientific Advisory Board, Sandler Program for Asthma Research
2001	Scientific Founder of AB Science, Paris, France
2002-	Member, Scientific Advisory Board, Synta Pharmaceuticals, Lexington, Massachusetts

Issued Patents:

United States Patent number: 5,639,660. Issued on June 17, 1997. Title: "Polypeptide and DNA sequence corresponding to the human receptor with high affinity for IgE"

United States Patent number: 5,770,396. Issued on June 23, 1998. Title: "Isolation, characterization, and use of the human beta subunit of the high affinity receptors for immunoglobulin E"

United States Patent number: 5,807,998. Issued on September 15, 1998. Title: "Isolation, characterization, and use of the □eta human and □amma subunit of the high affinity receptor for immunoglobulin E"

United States Patent number: 5,965,605. Issued on October 12, 1999. Title: "Inhibition of the binding human IgE to its receptor by tetracyclic compounds for the alleviation of IgE-mediated immune response"

United States Patent number: 6,165,744. Issued on December 26, 2000. Title: "Isolation and characterization of cDNAs coding for the alpha, beta., and gamma subunits of the high-affinity receptor for immunoglobulin E"

United States Patent number: 6,165,799. Issued on December 26, 2000. Title: "Detection of anti-Fc epsilonRI autoantibodies in asthmatics"

United States Patent number: 6,171,803. Issued on January 9, 2001. Title: "Isolation, characterization, and use of the human beta subunit of the high affinity receptor for immunoglobulin E"

United States Patent number: 6,423,501. Issued on July 23, 2002. Title: "Calcium-independent negative regulation by CD81 of receptor signaling"

United States Patent number: 6,602,983. Issued on August 5, 2003. Title: "Polypeptide and DNA sequence corresponding to human receptor with high affinity for IgE"

Major Research Interests:

Molecular and Cellular Biology of the Immune System;

Structure, Function and Biology of Immunoreceptors

Signal Transduction and Ions Channels in Cells of the Immune System

The Role of Mast cells in Health and Diseases

Bibliography

I. Original Reports

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2. Bataille, C., Kinet, J.-P., and Godon, J.P. Changes in renal venous pressure and parameters of renal function during paracentesis - concentration - reinjection of ascites. **Acta Gastroenterol. Belg.** *45*:122-123, 1982

3. Kinet, J.-P., Soyeur, D., Balland, N., Saint-Remy, M., Collignon, P., and Godon, J.P. Hemodynamic study of hypotension during hemodialysis. **Kidney Intern.** *21*:868-876, 1982

4. Kinet, J.-P., Frankenne, F., Hennen, G., Balland, N., Foidart, J., Saint-Remy, M., and Mahieu, P: Tumor necrosis induced by ex vivo perfusion of the plasma of adenocarcinoma patients through columns of protein A. **Bull. Mem. Acad. R. Med. Belg.** *138*:104-117, 1983

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6. Kinet, J.-P., Perez-Montfort, R., and Metzger H. Covalent crosslinking of subunits of the receptor for immunoglobulin E induced by immunoprecipitation. **Biochemistry** *22*:5729-5732, 1983

7. Alcaraz, G., Kinet, J.-P., Kumar, N., Wank, S.A., and Metzger, H. Phase separation of the receptor for IgE and its subunits in Triton X-114. **J. Biol. Chem.** *259*:14922-14927, 1984

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11. Kinet, J.-P., Hunt, J., Foidart, J.B., Desoroux, A., and Mahieu, P. Ex vivo perfusion of plasma over protein A columns in human mammary adenocarcinoma. Evidence for a Protein A leaking by radioimmunoassay. **Eur. J. Clin. Invest.** *16*:43-49, 1986

12. Kinet, J.-P., Bensinger, W.I., Balland, N., Saint Remy, M., Frankenne, F., Hennen, G., and Mahieu, P. Ex vivo perfusion of plasma over Protein A columns in human mammary adenocarcinoma. Role of the Fc binding capacity of protein A in the side effects and the tumoricidal response. **Eur. J. Clin. Invest** *16*:50-55, 1986

13. Solal-Celigny, P., Simeon, J., Herrera, A., Vingi, G., Bertrand, O., Mahieu, P., Kinet, J.-P., Raspaud, S., Sinegre, M., and Boivin, P. Effects of *ex vivo* plasma adsorption over Protein A sepharose in acute leukemia. **Leuk. Res.** *10*:643-649, 1986

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18. Kinet, J.-P., Blank, U., Ra, C., White, K., Metzger, H. and Kochan, J. Isolation and characterization of cDNAs coding for the β subunit of the high-affinity receptor for immunoglobulin E. **Proc. Natl. Acad. Sci. USA.** *85*:6483-6487, 1988

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II. Editorials, Reviews, Letters and Book Chapters

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Ion channels in the immune system as targets for immunosuppression

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The discovery of a diverse and unique subset of ion channels in T lymphocytes has led to a rapidly growing body of knowledge about their functional roles in the immune system. Potent and specific blockers have provided molecular tools to probe channel structure–function relations and to elucidate the involvement of K^+, Ca^{2+}, and Cl^- channels in T-cell activation and cell volume regulation. Recent advances in analyzing Kv1.3 channel structure–function relationships have defined binding sites for channel blockers, which have now been shown to be effective in suppressing T-cell function *in vivo*. Ion channels may provide excellent pharmaceutical targets for modulating immune system function.

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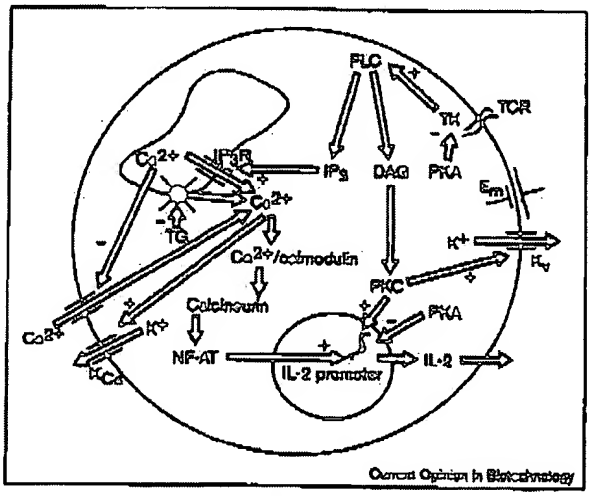
Abbreviations

$[Ca^{2+}]_i$	cytoplasmic Ca^{2+} concentration
CTX	charybdotoxin
IP_3	inositol 1,4,5-trisphosphate
K_V	voltage-gated K^+
K_{Ca}	calcium-activated K^+
PKC	protein kinase C

Introduction

The early stages of T-cell activation may be conceptually separated into pre-Ca^{2+} and post-Ca^{2+} events [1,2]. Initiated within 1–100 seconds of T-cell receptor engagement, pre-Ca^{2+} events include the activation of tyrosine kinases and the generation of inositol 1,4,5-trisphosphate (IP_3), leading to the release and influx of Ca^{2+} and the rise in cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$. Ranging from minutes to several hours, post-Ca^{2+} events involve changes in serine/threonine kinase and phosphatase activities, alterations in cytoskeleton and ion channel activity, and gene transcription. The rise in $[Ca^{2+}]_i$ activates the phosphatase calcineurin which then dephosphorylates a cytoplasmically localized transcription factor (nuclear factor of activated T cells) enabling it to accumulate in the nucleus and bind to a promoter element of the interleukin 2 gene. Along with other parallel events, involving the activation of protein kinase C (PKC) and ras, gene transcription leads to lymphokine secretion and to cell proliferation. A sustained or oscillatory Ca^{2+} signal results in dynamic changes in motility, morphology, and gene expression in T cells, with cytoskeletal elements and gene transcription exhibiting varying requirements for activation by $[Ca^{2+}]_i$; some genes requiring only a transient rise and others a long lasting or oscillatory Ca^{2+} signal [3,4,5°,6°]. Ca^{2+}-dependent immobilization of the T cell at the site of antigen presentation may help to cement the interaction between T cell and antigen-presenting cell and thereby facilitate the maintenance of local signaling between cells [5°]. Separate Ca^{2+}-dependent pathways control gene expression and motility; a pathway leading to interleukin 2 secretion is highlighted in Figure 1.

Figure 1



Signaling pathways in T cells. A signal transduction cascade leading from the T-cell receptor (TCR) to interleukin (IL)-2 secretion. DAG, diacylglycerol; E_m, plasma membrane potential; IP_3R, inositol 1,4,5-trisphosphate receptor; NF-AT, nuclear factor of activated T cells; PKA, protein kinase A; TG, thapsigargin; TK, tyrosine kinase. Plus signs indicate activation and minus signs indicate inhibition.

Presently, a post-Ca^{2+} target, calcineurin, is the site of action for immunosuppression. Unfortunately the calcineurin inhibitors cyclosporin A and FK506 are toxic, with liver and renal disease limiting their use. Thus, the search for additional immunosuppressive agents for transplantation or inflammatory diseases occupies considerable attention in the pharmaceutical industry. There is an excellent track record of treating nervous and cardiovascular disorders with channel modulators—either blockers or openers. Channel blockers, as a general class, represent the major therapeutic agents for treatment of stroke, epilepsy, and arrhythmia. These considerations suggest that ion channels may represent attractive sites

for pharmaceutical immunomodulation, targeting the pre-Ca^{2+} stage of activation. In this review, we consider Ca^{2+}-dependent signal transduction pathways that depend upon the activity of ion channels and survey progress in identifying and characterizing a surprisingly diverse and functionally significant population of ion channels in T cells.

Signaling pathways in T-cell activation: the role of ion channels

Ion channels underlie the Ca^{2+} signal of T cells [2]. Initially, phospholipase C-mobilized IP_3 produces a transient $[Ca^{2+}]_i$ rise by activating the IP_3 receptor, a Ca^{2+}-permeable ion channel located in the endoplasmic reticulum. IP_3 receptors have also been reported to reside in the surface membrane of T cells [7,8], but this result remains controversial. Functionally, IP_3 receptors, even if present in the plasma membrane, appear not to participate in the Ca^{2+} influx mechanism [9–11]. Ca^{2+} ions move across the plasma membrane through a Ca^{2+}-selective channel that is activated through an unknown mechanism by the depletion of Ca^{2+} from the endoplasmic reticulum [4,9,10,12–19]. Termed a store-operated Ca^{2+} channel, or a calcium release-activated Ca^{2+} channel, the lymphocyte Ca^{2+} channel is not gated by voltage, in contrast to voltage-gated Ca^{2+} channels found in neurons, muscle, and the heart. Once the Ca^{2+} channel is opened by store depletion, Ca^{2+} influx depends upon the plasma membrane potential to provide the electrical driving force to pull Ca^{2+} ions inward. Compared to conventional voltage-gated Ca^{2+} channels, the inverse dependence of Ca^{2+} influx on the plasma membrane potential is especially pronounced because current through the open Ca^{2+} channel rectifies inwardly.

Two distinct types of K^+ channels, a voltage-gated K^+ (K_V) channel and a Ca^{2+}-activated K^+ (K_{Ca}) channel, indirectly determine the driving force for Ca^{2+} entry [2]. When K^+ channels are open, the resulting efflux of K^+ drives the membrane potential to a negative voltage. The resting membrane potential of –50 to –60 mV in T cells is uniquely set by the voltage-gated channel (type *n* encoded by Kv1.3), which resists depolarization through its ability to open when the membrane is depolarized [20,21]. K_{Ca} channels are opened in a steeply cooperative manner by a rise in $[Ca^{2+}]_i$ following T cell receptor engagement, and these channels serve to hyperpolarize the membrane even further to –80 mV [20,22–25]. The hyperpolarization may accentuate Ca^{2+} influx in a positive feedback manner to promote the upstroke of the Ca^{2+} signal [26,27,28°].

Diversity of ion channels in T lymphocytes

In the early 1980s, development of the patch clamp technique and its unique applicability to a wide variety of unexplored cell types led to the first electrophysiological studies in T cells and to the identification of a K_V channel in resting human T cells [29–31]. Patch clamping permits electrical recording to identify and characterize ion channels with resolution to the level of single channel molecules. Several additional ion channels have since been characterized in T cells, as summarized in Table I.

K_V channels

Soon after the electrophysiological characterization of the dominant K_V channel and the identification of an array of chemically distinct K^+ channel blockers, it was discovered that these same channel-blocking agents are able to inhibit T-cell activation, including secretion of lymphokines, cell proliferation, and killing of target cells [29,32]. Two developments have greatly increased the pace of discovery regarding structure and function of the type *n* K_V channel. First, increasingly potent and selective peptide toxins are providing important tools for investigating the channel's functional role, for protein purification, for mapping the topology of the channel vestibule, and for high-throughput screens that have identified a new generation of highly potent blockers currently being pursued in the pharmaceutical industry [33,34,35°,36–46]. In 1989, two groups reported that peptide scorpion toxins can block the type *n* K_V channel at nanomolar concentrations [33,34]. At the time, the pharmacology of K^+ channels lagged behind other channel types, but now toxins have become important research tools and are also being explored for their therapeutic possibilities. In addition, the cloning of a series of K^+ channel genes and identification of Kv1.3 as the gene encoding the type *n* channel have facilitated drug discovery and structure-function relations [47–51]. The type *n* channel is a 65×65 Å homotetramer of Kv1.3 subunits, which contains several important functional domains that regulate channel gating, ion selectivity, and binding of drug molecules (Figure 2) [52–55].

Recently, the use of a peptide toxin as an immunosuppressant was validated by an *in vivo* mini-pig model of delayed-type hypersensitivity and allogeneic responses [56°°]. Margatoxin was found to be safe and significantly more potent than FK506 as an injectable immunosuppressant. Peptide toxins block the channel like a cork in a bottle, by binding to a 30 Å wide by 6 Å deep external vestibule, with several contact sites between the channel and the toxin molecule having been identified [36,37]. Mutagenesis of Kv1.3 has pointed to interactions between nonpeptidyl channel blockers, including dihydroquinolines and benzhydryl piperines for which considerable structure-activity information exists, and the inactivation gating mechanism which normally shuts the channel during prolonged depolarization [43]. A unique histidine in the outer mouth of the Kv1.3 channel, in addition to being involved in the inactivation gating mechanism, may confer selectivity upon the Kv1.3 channel for certain channel blockers [57–60]. Other classes of blockers, including verapamil, a classical Ca^{2+}-channel antagonist, may interact with residues near the inner channel vestibule [61,62]. The diversity of Kv1.3 channel blockers has recently been reviewed [44].

Table 1

Ion channels in lymphocytes.

Name	Gene	Conductance (pS)	Activation (midpoint)	Blockers (K_d range)	Expression (resting→proliferating)	Functional role
K_V						
n	Kv1.3	10–18	Voltage (–40 mV)	CTX nM MgTX nM TEA 10 mM	Human T cell (+→++++) Mouse T cell (+→++++) Mouse immature thymocytes +++ Jurkat +, numerous cell lines	Sets resting membrane potential Indirectly mediates Ca^{2+} influx RVD
l	Kv3.1	27	Voltage 0 mV	TEA 100 μM	Mouse $CD8^+$ thymocyte ++ *lpr, gld* $CD4^-CD8^-$ T cell +++	?
Other	Kv1.1?	?	Voltage –20 mV	DTX nM TEA 1 mM	Human T cell + Mouse thymocytes +, B3Z +	Supports membrane potential if Kv1.3 is blocked
K_{Ca}						
SK	SKCa3?	2–8	$[Ca^{2+}]_i$ 400 nM	Apamin pM TEA 2 mM	Jurkat +++	Hyperpolarization during Ca^{2+} signal promotes Ca^{2+} influx Ca^{2+}-dependent RVD
IK	IKCa1?	11–35	$[Ca^{2+}]_i$ 300 nM	CTX nM TEA 40 mM	Human T cell (+→++++) Mouse thymocytes ++	Mediates Ca^{2+} influx, oscillations
Ca^{2+}						
CRAC	TRP?	0.01	Ca^{2+} store depletion	La^{3+} nM Gd^{3+} nM	Jurkat +++ Human T cell Mouse thymocytes	
SWAC	?	?	Swelling	Gd^{3+} μM La^{3+} μM	Mouse immature thymocytes	Ca^{2+}-dependent RVD
P2X	P2X7?	?	μM ATP_o		Mouse thymocytes, T cells	Differentiation, activation?
Cl^-						
Maxi	?	2–3	Swelling ATP_i	DIDS μM NPPB μM	Human T cell (+++→++++) Mouse T cell (+++→++++)	Trigger for RVD

+ represents ~5–50, ++ 100–500, and +++ >500 channels per cell. CRAC, calcium release activated Ca^{2+} channel; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DTX, dendrotoxin; IK, intermediate conductance calcium-activated potassium channels; MgTx, margatoxin; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; RVD, regulatory volume decrease; SK, small conductance calcium-activated potassium channel; SWAC, swelling-activated Ca^{2+}-permeable channel; TEA, tetraethylammonium.

In general, the activity of ion channels can be modulated by direct channel block, phosphorylation or some other post-translational modification of the protein, or by altered levels of expression. The Kv1.3 channel is a substrate for protein kinase A, PKC, and tyrosine kinases in T cells; modulation of channel properties by phosphorylation may in turn impact signalling pathways involving the membrane potential [63–70]. Kv1.3 contains an adapter sequence at the carboxy terminus for the hDLG protein, which in turn associates with the tyrosine kinase p56*lck*, suggesting the existence of complexes of enzymes and channels at the membrane [71]. Functional effects of phosphorylation are still being clarified. Recently, attention has focused on tyrosine kinases, with papers documenting biochemical phosphorylation and decreased K^+ currents through Kv1.3 channels that could be relevant to fas-mediated apoptosis [66,67]. PKC stimulation was reported to decrease K^+ currents through Kv1.3 channels expressed in oocytes [63], but recently PKC stimulation has been reported to increase Kv1.3 currents in human T cells [64].

The tissue-specific and activation-dependent regulation of ion channel expression is poorly understood. The Kv1.3 gene contains an intronless coding region of 1.5 kilobases, yet mRNA species of 3.5–9.5 kilobases have been identified in lymphoid cells [47,51]. Regulatory elements controlling transcription have been described [72]. The levels of Kv1.3 expression are increased during T-cell activation, further suggesting a role in mitogenesis [2]. Kv1.3 is found associated with a β2 subunit which is also up-regulated during activation and may serve to stabilize the integrity of the channel complex [73].

Several groups have now identified additional K_V channels expressed at lower levels in lymphocytes, including charybdotoxin (CTX)-insensitive channels that may contribute to the maintenance of the membrane potential if Kv1.3 is blocked [25,74–76]. Inhibition of immune function by CTX and other toxins is generally not as complete as with less specific K^+ channel antagonists [2,27,34,39]. In murine T cells, another class of CTX-insensitive K_V channel, encoded by Kv3.1, is normally expressed in $CD8^+$ T cells, and aberrantly overexpressed in $CD4^-CD8^-$ T cells in several models of autoimmune disease, including lupus erythematosus, type 1 diabetes mellitus, collagen arthritis, and experimental allergic encephalomyelitis [2,77].

K_{Ca} channels

In addition to K_V channels, K_{Ca} channels are also attracting attention. Again, peptide toxins are providing useful tools for pharmacological separation of different channel components. The most common K_{Ca} channel in

Figure 2

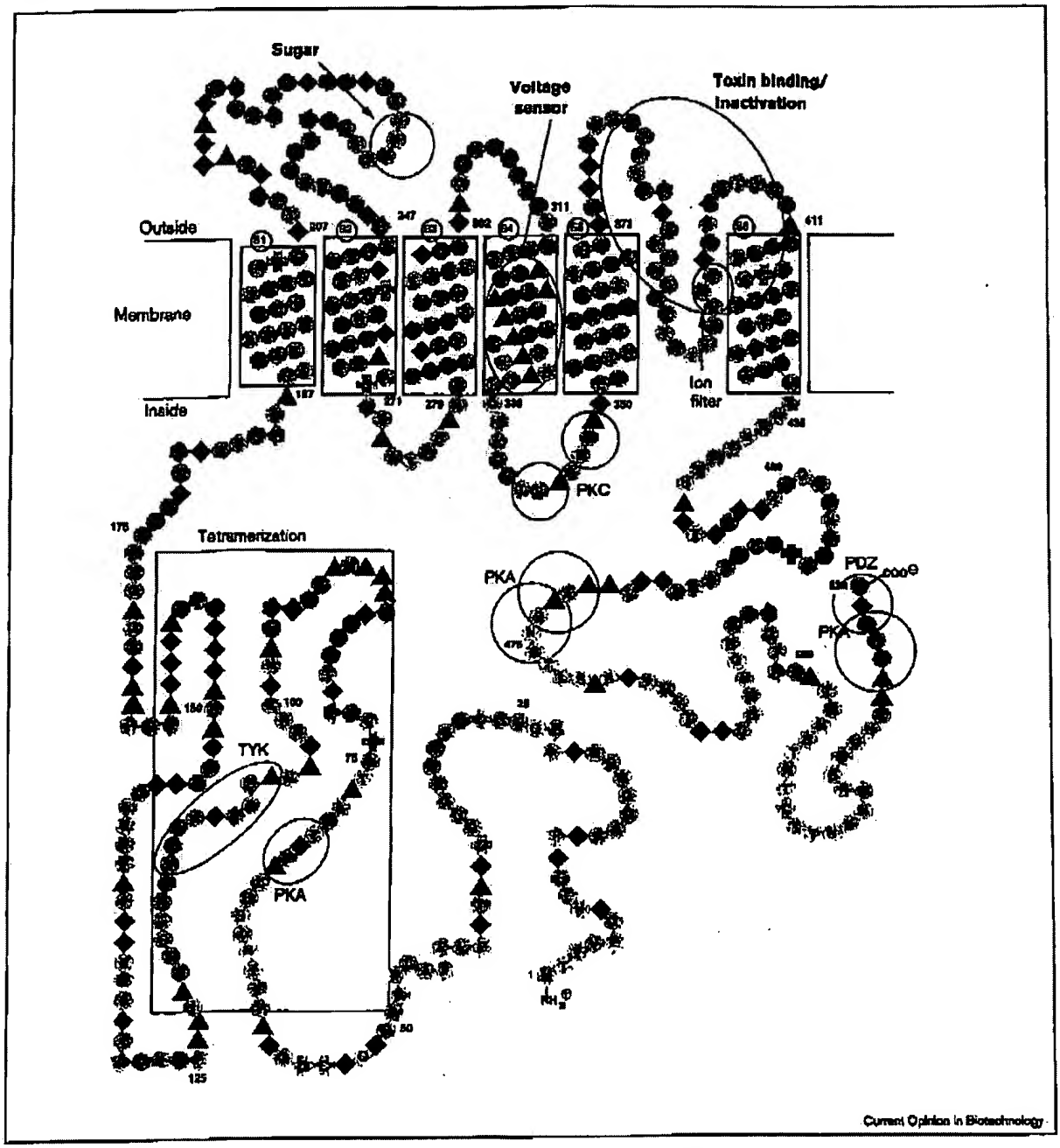


Diagram of Kv1.3. Major functional domains within the amino acid sequence (amino acid single letter code) of Kv1.3 are indicated. The channel is composed of four identical Kv1.3 subunits; the rectangle with 'tetramerization' shows the site of monomer–monomer interaction. The circles show sites that are functionally associated with various properties. S1, S2 etc., denote transmembrane segments. PKA, protein kinase A; TYK, tyrosine kinase.

human T cells is activated by a rise in $[Ca^{2+}]_i$ to 200 nM or more, has an intermediate single-channel conductance of 11–35 pS, and is blocked by nanomolar concentrations of CTX but not by margotoxin, kaliotoxin, or noxiustoxin, other toxins that block K_V channels at nanomolar levels or below [23]. Just to complicate matters, a commonly used T-cell lymphoma line (Jurkat) expresses a different, smaller conductance K_{Ca} channel that is blocked by

apamin but not by CTX, suggesting that transformation or lymphoid differentiation may alter their pattern of K_{Ca} channel expression [22,24]. In normal human T cells, expression of the intermediate conductance CTX-sensitive K_{Ca} channel is dramatically increased as T cells become activated to proliferate [23]. In parallel, activated T cells gain the ability to exhibit stronger and more oscillatory Ca^{2+} signals, raising the possibility that K_{Ca} channels participate indirectly by hyperpolarizing the membrane and thereby promoting Ca^{2+} entry [20,26,27,28°]. Targeting K_{Ca} channels may be particularly attractive in modulating autoimmune responses by previously activated T cells. Recently, a family of voltage-insensitive K_{Ca} channel genes has been discovered, encoding at least two small-conductance apamin-sensitive K_{Ca} channels and an intermediate conductance K_{Ca} channel [78°,79]. One of these, hIKCa1 or hSKCA4, is found in thymocytes and has properties similar to the intermediate conductance channel found in T cells [79,80]. The channel has been proposed to underlie the earliest known K_{Ca} channel — the Gardos channel in red blood cells, a therapeutic target for sickle cell anemia.

Ca^{2+} channels

Conceptually, the most direct target for toning down the $[Ca^{2+}]_i$ signal would be the Ca^{2+} channel [4,9,10–15]. With Ca^{2+} channels blocked, T cells exhibit only a meager and transient rise in $[Ca^{2+}]_i$; the Ca^{2+} channel is absolutely required for long-lasting signals that are capable of stimulating transcription. Furthermore, a novel primary T-cell immunodeficiency is associated with defective Ca^{2+} entry via Ca^{2+} channels [81]. Unfortunately, progress in identifying channel blockers for the Ca^{2+} channel has been slow, no peptide toxins or sub-micromolar blockers exist except for nonspecific, but surprisingly potent, La^{3+} ions [82,83]. Furthermore, the activation mechanism linking store depletion to channel opening, as well as the channels molecular identity, are still enigmatic. Similarities in ion permeation (but not gating) between voltage-gated and stores-operated Ca^{2+} channels suggest commonalities in pore-lining sequences and enable much larger monovalent currents to be studied [84].

Other channels, other functions

Several other channel types have been reported in patch-clamp and Ca^{2+} imaging experiments. Chloride channels activated by cell swelling were initially discovered in T cells but are also present in a variety of other cell types [85–91]. In lymphocytes, Cl^- channels provide the trigger for a homeostatic volume regulatory mechanism which restores the cell to its normal volume following exposure to a dilute environment [85,90]. Cell swelling in hypotonic solution activates the chloride channels, resulting in the loss of Cl^- and other permeable osmolytes, depolarization, and consequent opening of Kv1.3 channels. The loss of Cl^- and K^+ through their respective channels, along with osmotically obligated water, reduces cell volume. Cl^- channels may also play a role in mitogenesis by helping to maintain membrane potential [76,91]. In thymocytes, a calcium-permeable cation channel activated by cell swelling may complement this normally Ca^{2+}-independent mechanism for regulatory volume decrease, by providing a Ca^{2+}-dependent pathway and bringing K_{Ca} channels into play as a second K^+ efflux pathway [83].

Certain thymocyte subsets and mature T cells express P2Z receptors activated by extracellular ATP [92–98]. In the thymic microenvironment, ATP levels are probably high enough to activate these receptors, leading to the opening of a large Ca^{2+}- and cation-permeable channel. $[Ca^{2+}]_i$ signaling evoked by P2Z receptors has been implicated in triggering or enhancing proliferation, differentiation, and apoptosis. The role that these and other channels may play in lymphocyte function merits further investigation.

Conclusions

During the past few years, considerable attention has been focused upon T cell ion channels as targets for immunomodulation. A voltage-gated K^+ channel encoded by Kv1.3 has been subjected to the most intense scrutiny, with the effectiveness of highly selective peptide toxins in an *in vivo* model proving the concept that channel blockade can work to suppress the immune response. We can anticipate that channel blockers, by inhibiting pre-Ca^{2+} steps in the activation cascade may synergize with post-Ca^{2+} agents presently used, enabling doses of cyclosporin or FK506 to be substantially lowered, thereby reducing toxicity. The mix of ion channels expressed in a subset- and activation-dependent manner offers opportunities for continued development of channel blockers as immunomodulatory agents.

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Characterization of the cloned human intermediate-conductance Ca^{2+}-activated K^+ channel

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Jensen, Bo Skaaning, Dorte Strøbæk, Palle Christophersen, Tino Dyhring Jørgensen, Claus Hansen, Asli Silahtaroglu, Søren-Peter Olesen, and Philip Kiær Ahring. Characterization of the cloned human intermediate-conductance Ca^{2+}-activated K^+ channel. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C848–C856, 1998.—The human intermediate-conductance, Ca^{2+}-activated K^+ channel (hIK) was identified by searching the expressed sequence tag database. hIK was found to be identical to two recently cloned K^+ channels, hSK4 and hIK1. RNA dot blot analysis showed a widespread tissue expression, with the highest levels in salivary gland, placenta, trachea, and lung. With use of fluorescent in situ hybridization and radiation hybrid mapping, *hIK* mapped to chromosome 19q13.2 in the same region as the disease Diamond-Blackfan anemia. Stable expression of hIK in HEK-293 cells revealed single Ca^{2+}-activated K^+ channels exhibiting weak inward rectification (30 and 11 pS at −100 and +100 mV, respectively). Whole cell recordings showed a noninactivating, inwardly rectifying K^+ conductance. Ionic selectivity estimated from bi-ionic reversal potentials gave the permeability (P_K/P_X) sequence K^+ = Rb^+ (1.0) > Cs^+ (10.4) ≫ Na^+, Li^+, *N*-methyl-D-glucamine (>51). NH_4^+ blocked the channel completely. hIK was blocked by the classical inhibitors of the Gardos channel charybdotoxin (IC_{50} 28 nM) and clotrimazole (IC_{50} 153 nM) as well as by nitrendipine (IC_{50} 27 nM), *Stichodactyla* toxin (IC_{50} 291 nM), margatoxin (IC_{50} 459 nM), miconazole (IC_{50} 785 nM), econazole (IC_{50} 2.4 μM), and cetiedil (IC_{50} 79 μM). Finally, 1-ethyl-2-benzimidazolinone, an opener of the T84 cell IK channel, activated hIK with an EC_{50} of 74 μM.

intermediate-conductance calcium-activated potassium channel; charybdotoxin; clotrimazole; fluorescent in situ hybridization; radiation hybrid mapping; patch clamp; Diamond-Blackfan anemia

CALCIUM-ACTIVATED POTASSIUM channels are almost ubiquitously distributed in mammalian cells and constitute a major link between second messenger systems and the electrical activity of the cell. On the basis of their electrophysiological characteristics, three major classes of Ca^{2+}-activated K^+ channels have been described: voltage-dependent, large-conductance channels (BK); voltage-independent, small-conductance channels (SK); and inwardly rectifying, voltage-independent, intermediate-conductance channels (IK). BK (13) and SK (22) channels are widely distributed in excitable cells as well as in some nonexcitable cells (e.g., Ref. 13). In neurons, their activation underlies the generation of fast and slow afterhyperpolarizations, respectively, indicating a major role in the regulation of neuronal excitability. The human BK channel gene has been cloned from brain and functionally expressed in both *Xenopus* oocytes and mammalian cells (36). Three genes encoding the class of SK channels have been cloned from human brain (*hSK1*) and rat brain (*rSK2* and *rSK3*) (22). Through the use of the sequence information from these genes, human *IK* (*hIK1* or *hSK4*) has recently been cloned from placenta (21) and pancreas (20). The predicted amino acid sequence of hIK is related to but distinct from the neuronal SK channels.

IK channels are apparently absent in excitable tissues but are present in various blood cells (15), endothelial cells (31, 34), and cell lines of epithelial origin (7, 9). Physiologically, IK channels are strongly activated by release of intracellularly stored Ca^{2+}, induced by agonists such as ATP, bradykinin, and histamine. The activation of IK is followed by long-lasting or oscillatory hyperpolarizations of the cell membrane, which closely reflect the intracellular Ca^{2+} activity. In the present study, we define the chromosomal localization and describe the expression level of hIK in 50 different human tissues. The pharmacology and ion selectivity of the hIK channel after stable transfection of the gene into mammalian HEK-293 cells is examined.

MATERIALS AND METHODS

Cloning and Sequencing of hIK

A TBLASTN search of the GenBank database at the National Center for Biotechnology Information (NCBI) returned 12 human expressed sequence tag (hEST) sequences with homology to hSK1. Alignment of the sequences with hSK1 suggested that an hEST from placenta (GenBank no. N56819) could encode a full-length K^+ channel. This hEST was obtained from the American Tissue Culture Collection.

The cDNA of the hEST clone was sequenced bidirectionally by automated sequencing (ABI 377, Applied Biosystems) using a standard dideoxy chain-termination sequencing kit (Perkin-Elmer). Sequence assembly and analysis were carried out using Lasergene software (DNASTAR). The hEST clone contains a 1284-bp open reading frame, and when sequenced the hEST clone was found to correspond to *hIK1/hSK4* (20, 21).

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RNA Dot Blot Analysis

A ^{32}P-labeled cDNA probe was generated using random priming (Amersham, Little Chalfont, UK) and used to probe a commercially obtained human RNA master blot (Clontech, lot 7061008). Prehybridizations were for 30 min at 38°C in ExpressHyb (Clontech). Hybridization of the dot blot was for 16 h at 68°C with 10^9 dpm/μg probe in ExpressHyb. The blot was washed for 45 min at 25°C in 2× saline sodium citrate (SSC)-0.05% SDS, for 40 min at 50°C in 0.1× SSC-0.1% SDS, and finally for 30 min at 65°C in 0.1× SSC-0.1% SDS and was analyzed by autoradiography.

Radiation Hybrid Mapping

DNA from the subset of 86 GeneBridge4 clones for radiation hybrid (RH) mapping (18) was obtained from the Human Genome Mapping Project Resource Center (United Kingdom Medical Research Council). Primers (T-A-G-Copenhagen) located at positions 1474–1690 in the 3′ untranslated region of the *hIK* gene and amplifying a 238-bp fragment were used for RH mapping. PCR amplification was performed for 40 cycles in a PTC-225 DNA engine tetrad thermocycler (MJ Research) at 95, 59, and 72°C. PCR-generated fragments were separated on a 2% agarose gel, and amplification products of correct length were scored as positives, whereas absent products or PCR products of incorrect length were scored as ambiguities or negatives. Mapping was performed by direct submission to the RH mapping facility at The Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research.[1] After mapping, neighboring markers of the RH data vector were identified using the ENTREZ genome database at NCBI, and the chromosomal location was documented.

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization (FISH) with corresponding 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI)-banding and measurement of the relative distance from the long arm telomere to the signals [fractional length from the short arm telomere (FLpter value)] was performed essentially as described previously (25), using 100 ng biotin-labeled 1.9-kb *hIK*. The metaphases were visualized on a Leica DMRB epifluorescence microscope equipped with a Sensys 1400 charge-coupled device camera (Photometrics) and IPLab Spectrum imaging software (Vysis).

Stable Expression

hIK was excised from pT3T7 using *Eco*R I and *Not* I and subcloned into the mammalian expression vector pNS1Z (NeuroSearch), a custom-designed derivative of pcDNA3Zeo (InVitrogen). HEK-293 cells were grown in DMEM (Life Technologies) supplemented with 10% FCS (Life Technologies) at 37°C in 5% CO_2. One day before transfection, 10^6 cells were plated in a cell culture T25 flask (Nunc). Cells were transfected with 2.5 μg of the plasmid pNS1Z_hIK using Lipofectamine (Life Technologies) according to the manufacturer's instructions. Cells transfected with pNS1Z_hIK were selected in media supplemented with 0.25 mg/ml Zeocin. Single clones were picked and propagated in selection media until sufficient cells for freezing were available. Thereafter the cells were cultured in regular medium without selection agent. Expression of functional hIK channels was verified by patch-clamp measurements.

[1] RH mapping can be performed via the World Wide Web (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl).

Electrophysiology

Experiments were performed at room temperature with an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). Pipettes (1.5–3.0 MΩ) were pulled from borosilicate glass and, for single-channel experiments, coated with Sylgard and fire polished. A custom-made perfusion chamber (volume 15 μl) with a fixed AgCl-Ag pellet electrode was mounted on the stage of an inverted microscope equipped with Hoffman interference contrast.

A coverslip with transfected HEK-293 cells was transferred to the perfusion chamber and continuously superfused at a rate of 1 ml/min with an extracellular K^+ solution with a composition (in mM) of 144 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH 7.4). The pipettes were filled with intracellular solutions consisting of (in mM) 144 KCl, 10 HEPES, 1 or 10 EGTA, 9 or 0 nitrilotetraacetic acid, and $MgCl_2$ and $CaCl_2$ in the concentrations calculated (EqCal, Cambridge, UK) to give free Ca^{2+} concentrations of 0.1, 0.3, and 3 μM, respectively. Free Mg^{2+} concentration was always 1 mM, and pH was adjusted to 7.2 before experiments. These intracellular solutions were also used in the bath in inside-out experiments.

In the selectivity study, extracellular solutions consisting of 150 mM of the desired cation (Cl^- salts) and 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES [pH adjusted to 7.4 with 10 mM *N*-methyl-D-glucamine ($NMDG^+$)] were used.

The electrode potentials were always zeroed with the open pipette in a high-K^+ solution. No zero current or leak current subtraction was performed during the experiments. In whole cell experiments, the cell capacitance and the series resistance (R_s) were updated before each pulse application. R_s values ranged from 3 to 6 MΩ and remained stable during all experiments (10–30 min). At least 80% compensation of R_s was obtained. The current signals were low-pass filtered at 3 kHz (whole cell recordings) or 0.5–1 kHz (single-channel recordings) and digitized at a sample rate of at least three times the filter cutoff frequency. All analyses and drawings were performed with IGOR software (WaveMetrics, Lake Oswego, OR).

The IC_{50} values reported for the tested compounds were calculated from the kinetics of the block. The time course of the decrease in current (I) was fitted to the equation

$$I = I_0[1 - C/[C + (K_{off}/K_{on})]][1 - \exp[-(CK_{on} + K_{off})t]]$$

where K_{off} is off rate (in s^{-1}), K_{on} is on rate (in $M^{-1} \cdot s^{-1}$), I_0 is basal current, C is drug concentration, and t is time. $IC_{50} = K_{off}/K_{on}$, and this is the value that is reported.

Clotrimazole, charybdotoxin, iberiotoxin, apamin, econazole, and miconazole were from Sigma. Ketoconazole was from Research Biochemicals International. 1-Ethyl-2-benzimidazolinone (1-EBIO) was from Aldrich. Kaliotoxin, margatoxin, and *Stichodactyla* toxin were from Alomone Lab. Stock solutions were prepared in DMSO (clotrimazole, econazole, miconazole, ketoconazole, and 1-EBIO) or water (peptides) and diluted to final concentrations in the appropriate salt solutions. BSA (0.01% wt/vol) was present in all external solutions when peptides, clotrimazole, or clotrimazole analogs were used during the experiment. None of the vehicles had any effect on the recorded currents.

RESULTS

Cloning and Sequencing of hIK

The cloning of *hIK* was based on the identification of clones in the GenBank EST database with low but significant homology to hSK1. Although most of the

retrieved ESTs with homology to hSK1 were identical to or highly similar to the channels SK1, SK2, and SK3, the rest exhibited rather low homology (<50%) to known K^+ channels and were potentially members of a new branch of the six-transmembrane K^+ channel family. The majority of these ESTs are part of the IMAGE Consortium Washington University-Merck EST project (27). Three of the hESTs with low homology to hSK1 were obtained, and two pancreatic hESTs were found to be partial clones by sequencing. The last of these three hESTs was from placenta, and this cDNA clone includes a full-length open reading frame encoding a polypeptide of 427 amino acids. The initiating methionine was assigned to an in-frame ATG within a strong Kozak consensus site (GXXGCCATGG) (24) in the most 5′ end of the clone. The predicted molecular mass of hIK is 48 kDa.

Tissue Expression of hIK

Northern blot analysis of eight human tissues revealed a prominent 2.3-kb and a 2.9-kb transcript expressed in placenta (not shown). To estimate the tissue distribution of hIK, the expression of hIK in 50 different human tissues was examined by RNA dot blot analysis (Fig. 1). Significant amounts of transcript were evident in several nonexcitable tissues, including salivary gland, placenta, trachea, and lung, but not in any neuronal tissue (Fig. 1). The widespread distribution of hIK in tissues rich in epithelia suggests that hIK may represent an isoform primarily involved in secretion and absorption in salt-transporting epithelia.

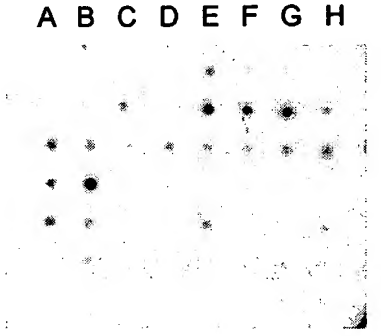
Chromosomal Localization of hIK

Mapping by RH analysis. To define the chromosomal localization of the *hIK* gene, PCR was performed on DNA from an RH panel that included 86 cell lines (18). Using RH mapping, the hIK-encoding gene was mapped to within 2.74 centiRays (lod score >3.0) of marker WI-6526 and centromeric to D19S420 (17) and is thus contained within chromosomal region 19q13.2–19q13.3.

Mapping by FISH. Specific FISH signals were observed on the distal part of the long arm of chromosome 19, with 58 of 60 analyzed metaphases (97%) displaying at least one specific signal. In total, 139 of the 240 chromatids (58%) were labeled; there were 2 cells with 0, 5 cells with 1, 28 cells with 2, 19 cells with 3, and 5 cells with 4 labeled chromatids. The FLpter value derived from measurements of 37 signal-bearing chromosomes was 0.132 ± 0.021, corresponding to a localization at 19q13.13–19q13.31 (3). The localization of the FISH signals on elongated metaphase chromosomes suggested a finer localization to the border of bands 19q13.13–19q13.2 (Fig. 2).

Stable Expression of hIK in HEK-293 Cells

Functional expression of hIK channels was revealed by single-channel as well as whole cell recordings. Figure 3*A* shows single-channel recordings from an inside-out patch exposed to symmetrical K^+-solutions with 0.3 μM free Ca^{2+} at the intracellular side. Single-channel openings were observed at both positive and negative membrane potentials, and the gating showed no significant voltage dependency. However, the channel exhibited clear open-channel inward rectification. In the experiment depicted in Fig. 3, the unitary inward current fluctuations estimated at −100 mV were 2.9 pA, whereas the corresponding outward currents were only 1.1 pA. The mean values of five independent experiments were 3.0 ± 0.2 and 1.1 ± 0.1 pA at −100 and +100 mV, respectively, corresponding



Human tissue	Detectable expression	Human tissue	Detectable expression
whole brain, H7	-	pancreas, F4	-
cerebellum, E7	-	pituitary gland, E4	-
cerebral cortex, D7	-	adrenal gland, D4	-
hippocampus, B7	-	thyroid gland, C4	-
substantia nigra, F6	-	salivary gland, B4	++++
10 other tissues from brain, B6,C6,D6,E6,G6,H6,A7,C7,F7,G7	-	mammary gland, A4	+
heart, H5	-	kidney, H3	++
aorta, G5	-	liver, G3	+
skeletal muscle, F5	-	small intestine, F3	+
colon, E5	+	spleen, E3	+
bladder, D5	-	thymus, D3	++
uterus, C5	-	peripheral leukocyte, C3	-
prostate, B5	+	lymph node, B3	+
stomach, A5	++	bone marrow, A3	++
testis, H4	-	appendix, H2	-
ovary, G4	-	lung, G2	+++
fetal liver, E1	+	trachea, F2	+++
6 other fetal tissues, B1,C1,D1,F1,G1,H1	-	placenta, E2	+++

Fig. 1. RNA dot blot analysis of expression of human intermediate-conductance, Ca^{2+}-activated K^+ channel (hIK) in human tissue. *Left*: human RNA master blot [2 μg of poly(A)$^+$-selected RNA, Clontech] was probed with a ^{32}P-labeled cDNA probe from hIK. *Right*: schematic representation of dot blot analysis of hIK expression in human tissues: +, detectable levels of transcript (relative expression indicated by no. of +); −, none detectable. Letters and numbers refer to columns and rows, respectively, of master blot at *left*.

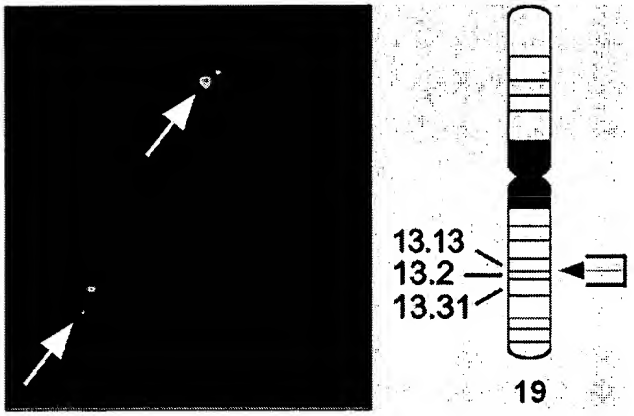


Fig. 2. Fluorescent in situ hybridization (FISH) mapping of *hIK*. *Left*: partial metaphase with specific FISH signals on 19q (arrows). *Right*: ideogram (12) showing localization of *hIK* based on DAPI-band pattern and mean FLpter value (arrow). Horizontal lines above and below arrow indicate variation in FLpter values among individual signal-bearing chromosomes.

to chord conductances of 30 ± 2 and 11 ± 1 pS, respectively. The single-channel current-voltage (*I-V*) relation is plotted in Fig. 3*B*.

Figure 4*A* shows a typical whole cell experiment with a slightly inwardly rectifying current developing during the first 30 s after break-in to the whole cell mode (Fig. 4*A*). Only a linear leak (*trace 1*) of a maximum of 100 pA was seen when a voltage ramp (−100 to +100 mV, 200 ms in duration) was applied at the very moment that the whole cell configuration was obtained. The hIK current (*trace 2* and *inset*) was activated as the cytosol was exchanged by the pipette solution due to the increase in intracellular Ca^{2+} concentration (3 μM in pipette solution). The reversal potential (V_r) shifted from 0 to −91 ± 3 mV (mean ± SE, n = 12; *trace 3*) on reduction of the extracellular K^+ concentration from 144 to 4 mM, indicating a high K^+/Na^+ selectivity (see also below). Furthermore, with the physiological ion gradient, the *I-V* curve became almost linear in the range from −100 to 50 mV, and a decrease in current was obtained at potentials >50 mV, as previously described for IK currents in human red blood cells (8).

Figure 4*B* shows the immediate appearance of a noninactivating whole cell hIK current as voltage steps were applied. The observed whole cell inward rectification therefore represents an instantaneous property of the channels (open channel rectification or voltage-dependent block) and is not related to voltage-dependent gating, in accordance with the single-channel data.

Selectivity of hIK

The selectivity of the hIK channel was addressed in bi-ionic whole cell experiments. Voltage ramps (−130 to +70 mV, 200 ms in duration) were applied, and control traces were recorded with the same high-K^+ solution in the pipette and in the bath. The bath solution was then changed to a solution containing 150 mM XCl, with X being Rb^+, Cs^+, Na^+, Li^+, NH_4^+, or $NMDG^+$. At the end of the experiment, 200 nM charybdotoxin was added to confirm the identity of the current. Figure 5 shows representative traces obtained with extracellular K^+ compared with those obtained in Rb^+, Na^+, Li^+, $NMDG^+$, Cs^+, and NH_4^+. Exchange of the bath solution from K^+ to Rb^+ did not alter the V_r (0 ± 0.3 mV, n = 15 for K^+; 1 ± 0.5 mV, n = 4 for Rb^+; means ± SE), indicating an equal permeability of K^+ and Rb^+. With extracellular Cs^+, the V_r shifted toward negative potentials, with a small but distinct (charybdotoxin-sensitive) inward current at potentials negative to −59 ± 1.4 mV (mean ± SE, n = 4), indicating a significant Cs^+ permeability. However, with Na^+, Li^+, or $NMDG^+$ in the bath, the V_r shifted from 0 mV to potentials more negative than −100 mV (often more than −130 mV, n = 3–7) within 30 s, indicating virtually no permeation of these ions. It was not possible to obtain an estimate of the permeability to NH_4^+, since this ion completely blocked the inward as well as the outward currents (n = 16). The permeability ratios (P_K/P_X) were calculated from the V_r values using the equation $P_K/P_X = \exp(-V_r F/RT)$, where F is Faraday's constant, R is the

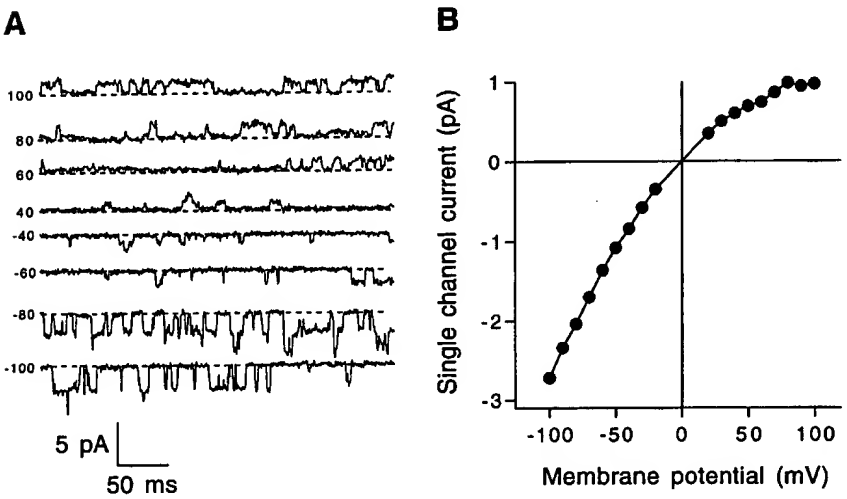


Fig. 3. *A*: hIK single-channel currents from an inside-out patch exposed to symmetrical (144 mM) K^+ solutions. "Intracellular" free Ca^{2+} concentration = 0.3 μM. Membrane potential was stepped from −100 mV to 100 mV in increments of 10 mV (15 ms at start and end of current traces was blanked to remove residual capacitive transients). Low-pass filtering: 500 Hz. *B*: single-channel current-voltage (*I-V*) curve obtained in symmetrical K^+-solutions.

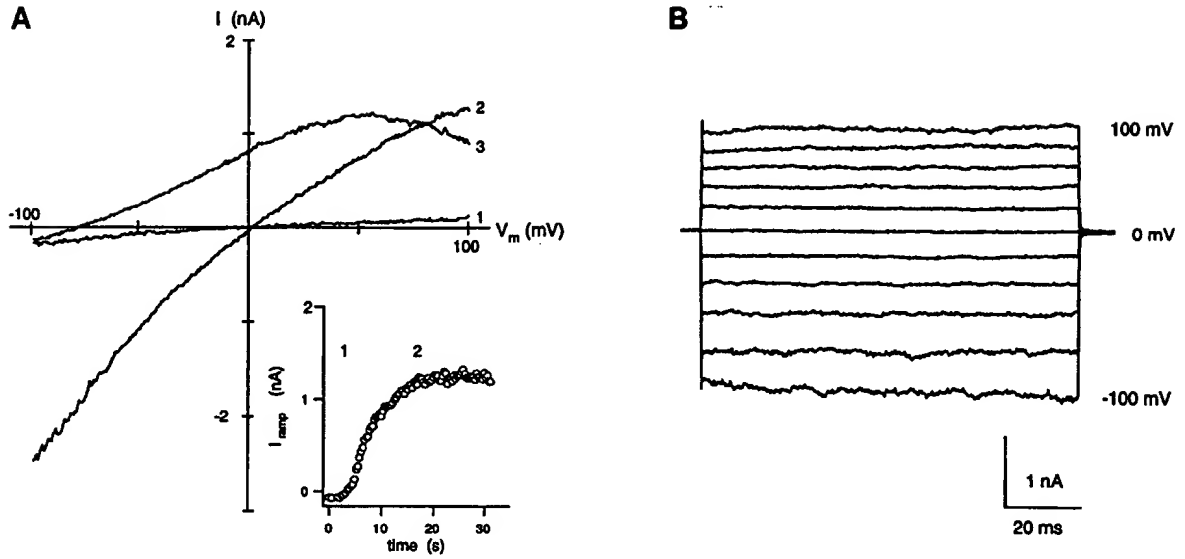


Fig. 4. *A*: whole cell currents (*I*) recorded on application of voltage ramps from −100 mV to +100 mV (200 ms in duration). Traces were recorded just after break-in (*trace 1*), 15 s later (*trace 2*), and after exchange of extracellular solution from a solution with 144 mM K^+ to a solution containing only 4 mM K^+ (*trace 3*). V_m, membrane potential. *Inset*: development of current (I_{ramp}) was measured at +80 mV from voltage ramps, and time course is shown, with 1 and 2 indicating times at which traces were obtained. *B*: currents recorded from another cell on application of voltage steps from −100 mV to +100 mV (20-mV increments, 100 ms in duration). Extracellular solution contained 144 mM KCl. Free Ca^{2+} concentration in pipette solutions was 3 μM in both *A* and *B*.

gas constant, and T is absolute temperature. The following permeability sequence was obtained: K^+ (1.0) = Rb^+ (1.0) > Cs^+ (10.4) ≫ Na^+, Li^+, $NMDG^+$ (>51). In the Eisenman theory for equilibrium selectivity, the observed sequence for the alkali metal ions corresponds to binding to a site of intermediate field strength (series III or IV).

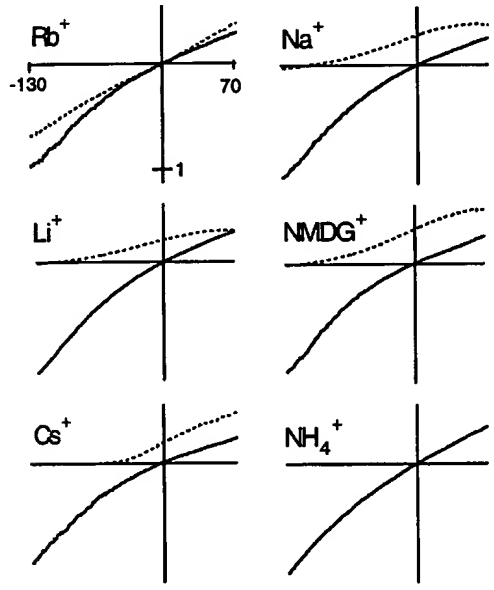


Fig. 5. Currents recorded on application of voltage ramps from −130 mV to +70 mV to transfected cells in whole cell configuration. Pipettes contained an intracellular high-K^+ solution with free Ca^{2+} concentration buffered to 300 nM. Each trace shows a new cell. Solid lines, traces obtained with K^+ solution in bath; dashed lines, traces recorded with indicated cations (150 mM) in bath. Note that dashed trace for NH_4^+ is almost covered by *x*-axis. Currents recorded in K^+ solution at −130 mV in individual cells were normalized for comparison.

Pharmacology of hIK

The sensitivity of the expressed hIK channel to three K^+ channel blockers is shown in Fig. 6. Ramp currents were elicited every 5 s, and the time course of the current at +80 and −80 mV is shown in Fig. 6*A*. Addition of 1 μM clotrimazole, a blocker of the IK channel in human erythrocytes (1), blocked the current in a reversible way. The SK channel inhibitor apamin (100 nM) was added for 2 min but failed to influence the hIK current. Finally, a nearly total block of the current was obtained after a shift to an extracellular solution containing 100 nM charybdotoxin. Figure 6*B* shows the control traces compared with the traces obtained after application of each of the three compounds for 1–2 min. IC_{50} values of 153 ± 33 nM (n = 11) for clotrimazole and 28 ± 3 nM (n = 19) for charybdotoxin were obtained. It is apparent from the time courses at ±80 mV as well as from the current traces that neither the block by clotrimazole nor the block by charybdotoxin was voltage dependent. The high sensitivity to block by charybdotoxin and clotrimazole, as well as the insensitivity toward the selective SK2 and SK3 channel blocker apamin, is consistent with the current being conducted by voltage-independent, Ca^{2+}-activated K^+ channels closely related to the human erythrocyte IK channel.

To strengthen this view further, we extended the pharmacological study to include *1*) other peptides with well-known K^+ channel blocker profiles, *2*) imidazole antimycotics considered to be analogs of clotrimazole,

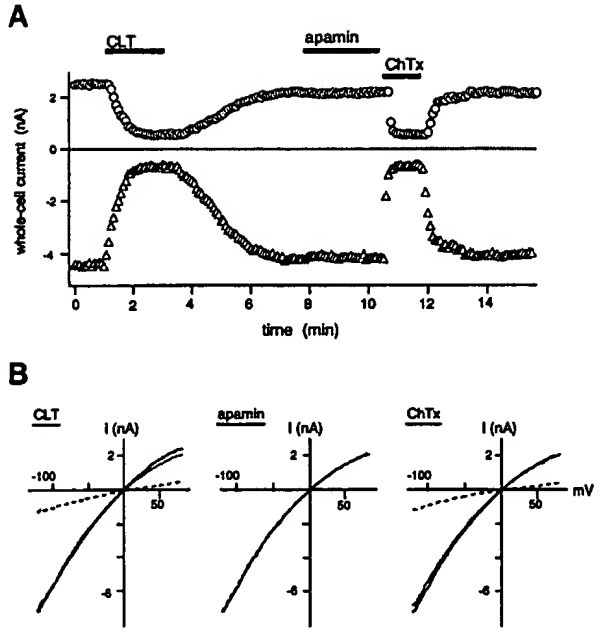


Fig. 6. *A*: time course of a whole cell experiment after application of clotrimazole (CLT; 1 μM), apamin (100 nM), and charybdotoxin (ChTx; 100 nM). hIK currents were measured at +80 mV (○) and at −80 mV (△) from traces obtained after application of voltage ramps. *B*: control (solid lines) and wash (dotted lines) traces, as well as traces recorded in presence of 3 compounds (dashed lines) after application of voltage ramps.

and *3*) various other compounds previously reported to modulate native IK channels. Figure 7 illustrates the pharmacological experiments. Among the peptides, *Stichodactyla* toxin (100 nM; Fig. 7*A*) and margatoxin (100 nM; Fig. 7*B*) blocked the current, although less potently than charybdotoxin. The mean IC_{50} values obtained were 291 ± 50 nM (n = 3) for *Stichodactyla* toxin and 459 ± 34 nM (n = 3) for margatoxin. Also shown in Fig. 7*B* is the lack of effect of the SK channel blocker dequalinium chloride (1 μM) (6). Furthermore, kaliotoxin (50 nM, n = 3) and iberiotoxin (300 nM, n = 5) had no discernible effects (Fig. 7*C*). This potency order is consistent with that obtained by Brugnara et al. (4) on Ca^{2+}-activated K^+ transport in erythrocytes, although the IC_{50} values obtained with erythrocyte suspensions were somewhat lower than the values obtained in the present study.

In addition to clotrimazole, the Ca^{2+}-dependent K^+ channels in human erythrocytes are blocked by several imidazole antimycotics (1). Figure 7*D* shows that hIK is blocked by econazole (IC_{50} = 2.4 ± 0.5 μM, n = 7), whereas ketoconazole is without effect (5 μM; n = 4). Miconazole blocked hIK with an IC_{50} = 785 ± 107 nM (n = 6).

Several classical Ca^{2+} channel antagonists were found to inhibit the Gardos channel (11), and the dihydropyridine nitrendipine is the most potent compound in this chemically diverse group of compounds. The effect of 300 nM nitrendipine is shown in Fig. 7*E*. The IC_{50} for the block by nitrendipine was 27 ± 6 nM (n = 6); nifedipine, a nitrendipine analog, was much less potent (1.5 ± 0.2 μM, n = 4), as were verapamil (72 ± 20 μM, n = 4) and diltiazem (154 ± 22 μM, n = 4).

Finally, cetiedil, a compound previously tested for its effect in sickle cell anemia, blocked the current with an IC_{50} value of 79 ± 44 μM (Fig. 7*F*; n = 4). Figure 7*F* also shows an activation of hIK by 10 μM 1-EBIO, which has been reported to activate Ca^{2+}-activated K^+ currents in colonic T84 epithelial cells and to stimulate short-circuit current and secretion in epithelia (10).

The activation of hIK by 1-EBIO was further elucidated by running cumulative dose-response experiments like the one shown in Fig. 8. In these experiments, the intracellular Ca^{2+} concentration was buffered at 0.1 μM, which is too low for significant activation of the channels. However, superfusion with 1-EBIO dose dependently activated the hIK current. The time course of a typical experiment is shown in Fig. 8*A*; the corresponding *I-V* curves are plotted in Fig. 8*B*. From the dose-response curve in Fig. 8*C*, an EC_{50} value of 74 ± 11 μM (n = 3) was estimated.

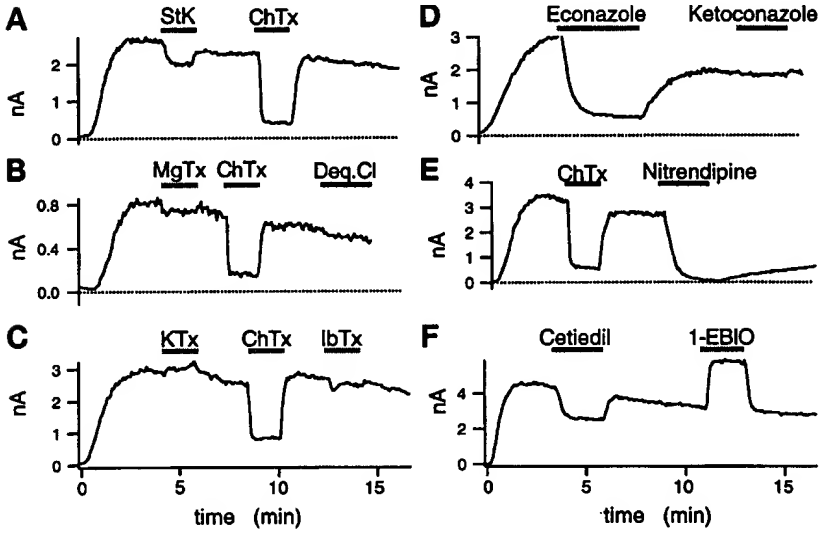


Fig. 7. *A–F*: time course of whole cell currents at +80 mV measured from voltage ramps (±100 mV every 5 s, 200 ms in duration) under control conditions (extracellular K^+ solution in bath) and during application of indicated compounds. Each trace represents a new cell. Concentrations used were 100 nM *Stichodactyla* toxin (StK), 50–100 nM charybdotoxin, 100 nM margatoxin (MgTx), 1 μM dequalinium chloride (Deq.Cl), 50 nM kaliotoxin (KTx), 300 nM iberiotoxin (IbTx), 5 μM econazole, 5 μM ketoconazole, 1 μM nitrendipine, 100 μM cetiedil, and 10 μM 1-ethyl-2-benzimidazolinone (1-EBIO). Free Ca^{2+} concentration in pipette solutions was 300 nM, and increase in currents apparent at beginning of each trace is activation of current by Ca^{2+} in pipette solution.

DISCUSSION

In summary, we have cloned, expressed, and functionally characterized an hIK from human placenta. The gene was localized at chromosome 19 at region 19q13.2–19q13.3, and mRNA expression was detected at the highest density in salivary gland, placenta, lung, and trachea and in smaller quantities in liver, colon, thymus, kidney, and bone marrow. It was absent from excitable tissues, such as brain and skeletal and heart muscle, as well as from most embryonic tissues except liver. Recently, data on the cloning and expression of a pancreatic IK channel with similar characteristics were presented by Ishii et al. (20), whereas Kaczmarek and co-workers (21) designated the very same channel hSK4 due to the similarity of hIK to the SK channel family (~45% identity on the amino acid level). However, the functional and pharmacological characteristics, as well as the tissue distribution of hIK expression, strongly suggest that the cloned channel is identical to the well-defined type of IK channel present in a number of nonexcitable cells, such as T and B lymphocytes (14, 32), red blood cells (8, 15), and cells derived from epithelia (7, 35) and endothelia (34). Logsdon et al. (28) recently cloned an IK channel from T lymphocytes that is identical to hIK.

The unifying functional characteristics of this group of native channels are the single-channel conductance, which exhibits weak inward rectification in symmetrical K^+ (30–40 pS at −100 mV; 10–15 pS at 100 mV), the weak or absent voltage dependency of gating, and the high and steep sensitivity to intracellular Ca^{2+} (8, 10, 15, 31, 35). In comparison, the estimated single-channel chord conductance for the cloned channel is 30 pS (present study) or ~33 pS (20).

The cloned channel was found to be highly selective for K^+, with demonstrable permeability (P_X/P_K) to Rb^+ (1.0) and Cs^+ (10.4), whereas Li^+, Na^+, and $NMDG^+$ were largely impermeable (>51). The permeability sequence obtained for hIK closely resembles that of the prototype channel within this group, the erythrocyte K^+ channel (8). The notable differences are *1*) that Cs^+ in the present study was demonstrated to conduct inward current, *2*) the powerful block of the cloned channel by NH_4^+, and *3*) a tendency of hIK toward decreased selectivity after prolonged exposure to K^+-free solutions. Regarding *difference 1*, inward single-channel currents conducted by Cs^+ were impossible to demonstrate in the erythrocyte channel study, most likely due to very small single-channel currents. However, the extrapolated value of the V_r in that study corresponds to the value determined here. Regarding *difference 2*, the erythrocyte channel was not completely blocked by NH_4^+, and genuine permeability measurements were possible at the single-channel level (P_K/P_X = 8.5). However, the gating of the erythrocyte channel was pronouncedly changed in NH_4^+ solutions (short openings, low open state probability) (2). Qualitatively, the complete block seen in the present study may reflect the same phenomenon. Regarding *difference 3*, after a longer period in Na^+, Li^+, or $NMDG^+$, the decrease of the outward current at positive potentials became more prominent, a small inward current developed at very negative potentials, and the V_r shifted toward less negative potentials. This could indicate that the channels become permeable to these ions in the total absence of external K^+, as described by Korn and Ikeda (23) for Kv1.5. However, although mechanistically interesting, the phenomenon has not been further elucidated in this study.

In addition to the difference in open channel properties, the SK and IK channels can be distinguished pharmacologically. It is well known that charybdotoxin

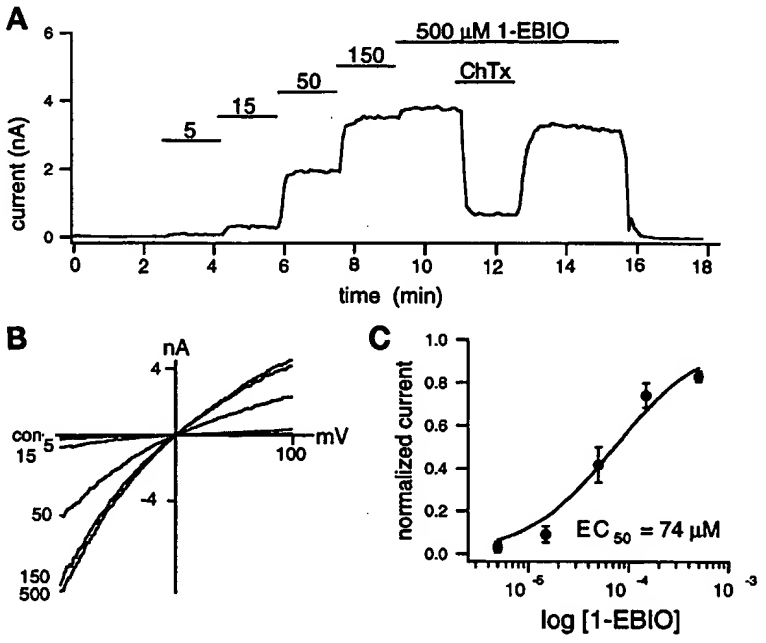


Fig. 8. *A*: time course of whole cell current as in Fig. 7. *B*: corresponding whole cell currents measured with 0 (control; con), 5, 15, 50, 150, and 500 μM 1-EBIO in bath. *C*: mean dose-response relationship from 3 cells.

blocks IK channels with high affinity (low nanomolar range, e.g., Ref. 4), whereas SK channels are not affected. Conversely, apamin blocks certain SK channels (equivalent to the cloned SK2 and SK3), whereas there is no effect on IK channels. Because the cloned hIK is potently blocked by charybdotoxin and is insensitive to apamin [and to dequalinium, which binds to the apamin-site (6)], the basic pharmacology points toward a classical IK channel. The experiments with the K^+ channel-blocking peptides clearly show that charybdotoxin is the most potent, with a potency order exactly as described for erythrocytes. Clotrimazole, nitrendipine, cetiedil, and 1-EBIO are classical modulators of IK channels, but their selectivity properties are largely unknown. However, the overall correlation between the potency of these compounds (and their analogs) on native IK channels and the cloned channel supports the conclusion of a closely related pharmacology.

The physiological significance of IK channels has been described in various tissues. The erythrocyte K^+ channel was the first Ca^{2+}-activated K^+ channel ever described, but despite this the function of IK channels in normal erythrocytes is obscure, whereas pathological activation is known to be critical in the development of crises in sickle cell anemia (5). The *hIK* gene is mapped to chromosome 19q13.2 at the same localization as Diamond-Blackfan anemia (16), a disease with the clinical hallmarks of a selective decrease in erythroid precursors and anemia. In some salt (and water) secreting epithelia, activation of basolateral IK channels is essential for maintaining the driving force for luminal Cl^- efflux (10). In the lung, where hIK is expressed relatively abundantly, a charybdotoxin-sensitive IK channel is thought to hyperpolarize the airway epithelia in response to agonists that elevate intracellular cAMP and Ca^{2+}, thus facilitating apical Cl^- secretion (29, 37). Possibly a pharmacological activator of hIK might therefore be of clinical interest in the treatment of mild forms of cystic fibrosis. Furthermore, hIK is expressed in colon (Fig. 1), and activation of Ca^{2+}-activated K^+ channels has been shown to be coupled to the apical Cl^- secretion (9, 33). Blockers of hIK may thus prove efficient in the treatment of secretory diarrhea. The high level of mRNA expression detected in salt-transporting tissues (Fig. 1) may indicate a universal role of IK channels in epithelial function. In T and B lymphocytes, the number of IK channels is upregulated on activation with antibodies or the protein kinase C activator phorbol 12-myristate 13-acetate (14, 32), suggesting important functions of this class of channels in the activation of the immune system.

A number of native channels with characteristics different from those of the erythrocyte-type IK channel have traditionally been classified as IK channels. Thus the IK channel group is quite heterogeneous, comprising channels that differ widely in single-channel conductances (20–150 pS), degree of rectification, voltage dependency of gating, pharmacology, and Ca^{2+} sensitivity (19, 26, 30). Many of these channels deviate strongly from the channel described in the present paper, and some are even expressed in excitable cells. It is therefore possible that a whole family of IK channels exists and that more types of Ca^{2+}-activated K^+ channels with intermediate conductance will be cloned in the future.

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Physiology

Inhibition of T cell proliferation by selective block of Ca^{2+}-activated K^+ channels

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ABSTRACT **T lymphocytes express a plethora of distinct ion channels that participate in the control of calcium homeostasis and signal transduction. Potassium channels play a critical role in the modulation of T cell calcium signaling, and the significance of the voltage-dependent K channel, Kv1.3, is well established. The recent cloning of the Ca^{2+}-activated, intermediate-conductance K^+ channel (IK channel) has enabled a detailed investigation of the role of this highly Ca^{2+}-sensitive K^+ channel in the calcium signaling and subsequent regulation of T cell proliferation. The role IK channels play in T cell activation and proliferation has been investigated by using various blockers of IK channels. The Ca^{2+}-activated K^+ current in human T cells is shown by the whole-cell voltage-clamp technique to be highly sensitive to clotrimazole, charybdotoxin, and nitrendipine, but not to ketoconazole. Clotrimazole, nitrendipine, and charybdotoxin block T cell activation induced by signals that elicit a rise in intracellular Ca^{2+}—e.g., phytohemagglutinin, Con A, and antigens such as *Candida albicans* and tetanus toxin in a dose-dependent manner. The release of IFN-γ from activated T cells is also inhibited after block of IK channels by clotrimazole. Clotrimazole and cyclosporin A act synergistically to inhibit T cell proliferation, which confirms that block of IK channels affects the process downstream from T cell receptor activation. We suggest that IK channels constitute another target for immune suppression.**

All mammalian cells express potassium (K^+) channels in their cell membranes, and the channels play a dominant role in the regulation of the membrane potential. The intermediate-conductance, Ca^{2+}-activated K^+ channels (IK) are present in various blood cells, endothelial cells, and epithelial cells, but not in excitable tissue. Expression of IK channel mRNA has been established in a number of tissues—e.g., spleen, thymus, peripheral blood leukocytes, and T cells (1–3).

Physiologically, IK channels are important for Cl^- secretory epithelia, and for activation of T cells. Release of intracellularly stored Ca^{2+}, induced by agonists such as ATP, results in pronounced activation of IK channels. The activation of IK channels is followed by long-lasting or oscillatory hyperpolarizations of the cell membrane. The hyperpolarizations closely reflect the activity of intracellular Ca^{2+}. The IK channel (*i*) is highly K-selective; (*ii*) is activated by submicromolar concentrations of Ca^{2+}; (*iii*) has an inwardly rectifying conductance; and (*iv*) has as the calcium sensor calmodulin, which is constitutively bound to the channels (4). In T lymphocytes, the opening of the IK channels after the initial antigen stimulation of the T cell receptor hyperpolarizes the membrane and increases the influx of calcium. Furthermore, both the amount of mRNA and the number of expressed IK channels increases by 15- 20-fold upon activation of the T lymphocytes, indicating an important role for IK channels during lymphocyte proliferation (3). Previous studies have indicated the importance of Kv1.3 channels in hyperpolarizing the membrane potential to maintain an inwardly directed driving force for the secondary influx of calcium from the extracellular medium (5). Here we provide data suggesting that IK channels dominate the K^+ conductance of the activated T cell, and thus are responsible for providing the electrochemical driving force for Ca^{2+} entry into T cells. This idea, that inhibition of IK channel activity could decrease Ca^{2+} influx and subsequent T cell proliferation, has been evaluated in the present study.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PMBC) were obtained from healthy volunteers as described (6). In six experiments, T cells were depleted of plastic-adherent cells and purified by using nylon wool separation as described (7). All antigen $CD4^+$ T cell lines have been characterized previously (8).

Electrophysiology. The patch-clamp experiments were performed as described previously (2).

Salt solutions. (*i*) Extracellular. High-K^+ solution: 150 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% BSA, and 10 mM Hepes (pH 7.4). (*ii*) Intracellular solution: 150 mM KCl, 1 mM EGTA, 9 mM nitrilotriacetic acid, and 10 mM Hepes (pH 7.2). Solutions of 0.955 mM $CaCl_2$ and 5.585 mM $MgCl_2$ were added to obtain final free concentrations of 1 μM and 1 mM, respectively, as calculated with EQCAL software (Biosoft, Cambridge, U.K.). Clotrimazole, nitrendipine, and ketoconazole were purchased from Sigma. Charybdotoxin was from Alomone Labs, Jerusalem, Israel. Stock solutions were prepared in DMSO (organic compounds) or in extracellular solution (charybdotoxin) and added directly to the relevant experimental solution. All chemicals were of the purest grade commercially available.

Experimental procedure. Coverslips containing resting or activated T cells were placed in a perfusion chamber and continuously superfused with extracellular salt solution. In the whole-cell voltage-clamp experiments the series resistance as well as the cell capacitance were followed on-line and compensated by 70%. Initial series resistances were below 5 MΩ and usually remained constant throughout the experiments. Experiments were invariably discharged if series resistance increased above 10 MΩ or if slow capacitance cancellation failed. All experiments were performed at room temperature (21–25°C).

Proliferation Assays. Assays were performed in culture medium (RPMI medium 1640; GIBCO) supplemented with

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Abbreviations: CRAC channel, calcium-release activated Ca^{2+} channel; IK channel, Ca^{2+}-activated intermediate-conductance K^+ channel; PPD, purified protein derivative; PHA, phytohemagglutinin.

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10% fetal calf serum, 2 mM L-glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin (Novo, Copenhagen) in 96-well round-bottom tissue culture plates (Nunc) in a final volume of 200 μl. PBMC or T cells plus irradiated plastic-adherent cells (APC) were cultured at 5×10^4 cells per well for 5 days with or without reagents as indicated. Twelve hours before harvest, [^{3}H]thymidine (1 mCi per well; 1 Ci = 37 GBq) was added. The cells were harvested onto glass fiber filters, [^{3}H]thymidine incorporation was measured in a scintillation counter, and the results were expressed as median cpm from triplicate cultures (6–8).

Cytokine Release Assay. Cells were incubated for 24 or 48 hr with or without antigens in a humidified atmosphere as above. Harvest of the supernatants was carried out after pelleting the cells. IFN-γ was measured by using IFN-γ-specific ELISA as described elsewhere (9).

RESULTS

Electrophysiology. Whole-cell recordings of Con A-activated T cells revealed the expression of IK channels in activated T cells. A typical whole-cell experiment exhibited a slightly inwardly rectifying current (150–1000 pA) in activated T cells (not shown). In contrast, only a linear current of maximally 10 pA was observed when a voltage ramp (−100 to +100 mV, 200 ms in duration) was applied to a resting T cell. The sensitivity of the endogenous IK channel in activated T cells to clotrimazole, ketoconazole, nitrendipine, and charybdotoxin is shown in Fig. 1. Ramp currents were elicited every 5 s, and the time course of the current at −80 mV is shown (Fig. 1). Clotrimazole (1 μM), a blocker of both endogenous and cloned IK channels (1–3), blocked the current fully (n = 4). Charybdotoxin (200 nM), a peptide blocker of both Kv1.3 and IK channels, fully inhibited the IK current in a potent and reversible way (n = 4), as did nitrendipine (1 μM, n = 3). Ketoconazole (1 μM) had no effect on the IK current in T cells (Fig. 1, n = 3). These results correspond closely to those obtained on the cloned IK channel (2).

T Cell Proliferation. Using the three most potent blockers of the cloned IK channel, clotrimazole, charybdotoxin, and nitrendipine, we investigated the effect of IK channel block on T cell proliferation. Activation of T cells upon antigen stimulation is strongly inhibited when clotrimazole is used as IK channel blocker (Fig. 2). Clotrimazole is not a selective compound, and in addition to its effects on IK channels it also blocks cytochrome P450 and calcium-release-activated Ca^{2+} (CRAC) channels. Therefore, we also studied the closely related compound ketoconazole, which in contrast to clotrimazole is a more potent blocker of CRAC channels than of IK channels.

Fig. 2*A* shows the antigen-induced T cell proliferation in the presence of vehicle (control), 10 μM ketoconazole, and 10 μM clotrimazole. The T cell proliferation was estimated as the [^{3}H]thymidine incorporation 6 days after antigen stimulation. T cell activation in the presence of ketoconazole was only slightly inhibited compared with controls (23% ± 3%). In contrast, nitrendipine (10 μM), clotrimazole (10 μM), and

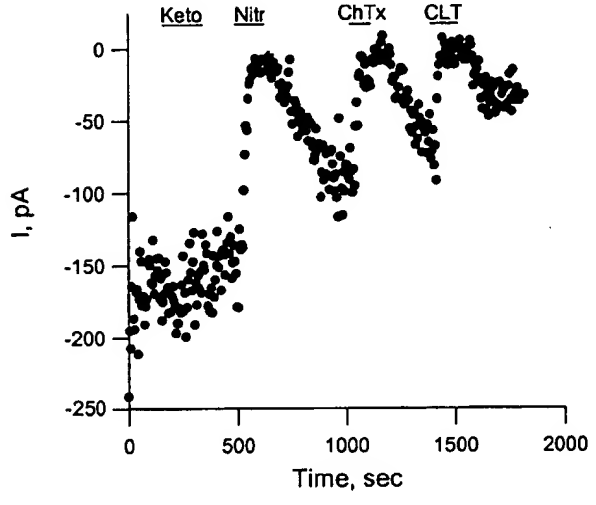

FIG. 1. Time course of whole-cell currents from a T cell activated by purified protein derivative (PPD) at −80 mV measured from voltage ramps (±100 mV every 5 s, 200-ms duration) under control conditions (extracellular K^+ solution in the bath) and during the application of the indicated compounds. The concentrations used were ketoconazole (Keto), 1 μM; nitrendipine (Nitr), 1 μM; charybdotoxin (ChTx), 200 nM; and clotrimazole (CLT), 1 μM. Free Ca^{2+} in the pipette solutions was 1 μM.

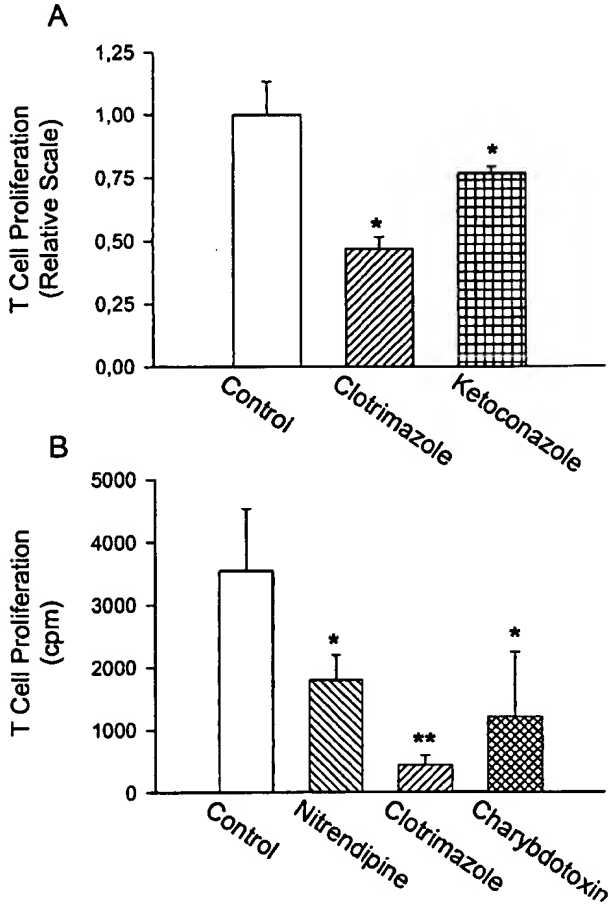

FIG. 2. (*A*) Effect of clotrimazole and ketoconazole on T cell proliferation. Cells were incubated for 5 days in culture medium with PPD and the IK channel blockers (10 μM clotrimazole or 10 μM ketoconazole), which were added 30 min before the addition of PPD. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents 12 independent experiments ± SE; control = $26 \pm 12 \times 10^3$ cpm per well; clotrimazole = $12 \pm 6 \times 10^3$ cpm per well; ketoconazole = $20 \pm 10 \times 10^3$ cpm per well. *, $P \leq 0.008$ vs. control. (*B*) Effect of nitrendipine, clotrimazole, and charybdotoxin on T cell proliferation. Cells were incubated for 5 days in culture medium with *Candida albicans* and the IK channel blockers (10 μM nitrendipine, 100 nM charybdotoxin, or 10 μM clotrimazole), which were added 30 min before the addition of *Candida albicans*. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents 9 independent experiments ± SE for control, and 3 independent experiments in the presence of nitrendipine, clotrimazole, and charybdotoxin, respectively. *, $P \leq 0.05$ vs. control; **, $P \leq 0.01$ vs. control.

charybdotoxin (100 nM), all IK channel blockers, decreased T cell proliferation significantly (Fig. 2*B*). In the experiments shown, charybdotoxin inhibited T cell proliferation by 67%, nitrendipine by 50%, and clotrimazole by 89%.

The potency of the IK channel blockers was investigated further. A dose–response relation of IK channel block on T cell proliferation after antigen stimulation is shown in Fig. 3 *Upper*. T cell proliferation is increasingly inhibited by clotrimazole, and even at 10 μM, maximal inhibition is not yet accomplished. Both nitrendipine and charybdotoxin appear to be less potent and exert their maximal effect at a concentration of 5 μM and 10 nM, respectively (Fig. 3 *Upper*). This indicates that clotrimazole inhibits T cell proliferation by additional means other than IK channel block, maybe by interference with the intracellular Ca^{2+} store system as suggested previously for human melanoma cells (10). Taken together, the data for the IK channel blockers suggest that maximal inhibition of IK channels accounts for a 40–50% inhibition of the T cell proliferation.

During the course of the present study, a significant donor and stimulus variation was observed. For the T cell proliferation induced by mitogens the inhibition by 10 μM clotrimazole varied from 10% to 84% in the 14 donors studied, whereas the antigen-induced T cell proliferation was inhibited by 20–100% in the presence of 10 μM clotrimazole. An example of the varied suppression by the T cell response to different donors and proliferative compounds is shown in Fig. 3 *Lower*. T cells from three different donors respond differently; however the inhibition of the T cell proliferation is significant in T cells from all three donors, as clotrimazole at 10 μM inhibits approximately 40–80% of the proliferation response. Clotrimazole at 1 μM is ineffective except for donor 3. Clotrimazole inhibits antigen-mediated T cell proliferation by 50–90% when antigens such *Candida albicans*, PPD, and tetanus toxin are used.

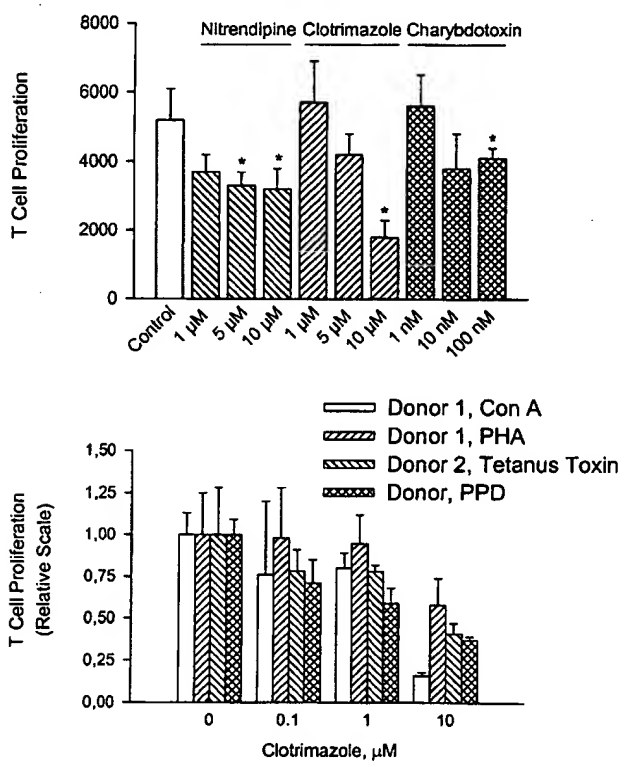

FIG. 3. (*Upper*) Dose–response relations of nitrendipine, clotrimazole, and charybdotoxin on T cell proliferation. Cells were incubated for 5 days in culture medium with PPD. The IK channel blockers, in the concentrations indicated, were added 30 min before the addition of antigen. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents nine independent experiments ± SE for control, and three independent experiments in the presence of nitrendipine, clotrimazole, or charybdotoxin, respectively. *, $P \leq 0.05$ vs. control. Six other experiments using *Candida albicans* or tetanus toxin as the antigen challenge gave similar results. (*Lower*) Donor variability of the clotrimazole effect on T cell proliferation. PHA, phytohemagglutinin. Cells from three different donors were incubated for 5 days in culture medium with mitogen (ConA or PHA) or antigen (tetanus toxin or PPD) and 10 μM clotrimazole, which was added 30 min before the addition of *Candida albicans*. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents three independent experiments ± SE.

Clotrimazole (10 μM) inhibits the T cell proliferation responses to alloantigens in different T cell subsets such as $CD4^+$, $CD45RA^+$, and CD45RO T cells, whereas no effect is observed in an Epstein–Barr virus-transformed B cell line (results not shown). Activation of T cells by bacterial superantigens, such staphylococcal enterotoxins (SEA and SEE) is inhibited by 10 μM clotrimazole. Clotrimazole inhibits expression of CD25 and of the MHC II complex, but has no effect on expression of CD45 (results not shown). The reason for the different sensitivity of antigen- and mitogen-induced T cell proliferation to IK channel block is not obvious. It might indicate different pathways of T cell activation, the antigen-induced response involving the inositol trisphosphate/calcium signaling pathway, and the mitogen-induced pathway being more broad in its mechanism of T cell activation, including both Ca^{2+}-dependent and Ca^{2+}-independent activation pathways.

The inhibition of T cell proliferation is seen at a clotrimazole concentration of 5 μM, which is above the concentration needed to block the IK current as shown in Fig. 1. The reason is that to inhibit the hyperpolarization completely most of the channels must be blocked. We have studied in detail the relationship between IK current and membrane potential on cloned IK channels by using blockers as well as openers of IK channels at different Ca^{2+} levels (ref. 11 and unpublished results). Briefly, the conclusion is that depolarizations are obtained when blockers are used in concentrations in excess of 5 times the IC_{50} value, but the result depends on the internal Ca^{2+} level, which may be the explanation for the variation obtained with different donors and stimuli.

Fig. 4 shows the release of IFN-γ in unstimulated T cells and in T cells stimulated by PPD in the presence of vehicle or clotrimazole (10 μM). Clotrimazole inhibits the release of

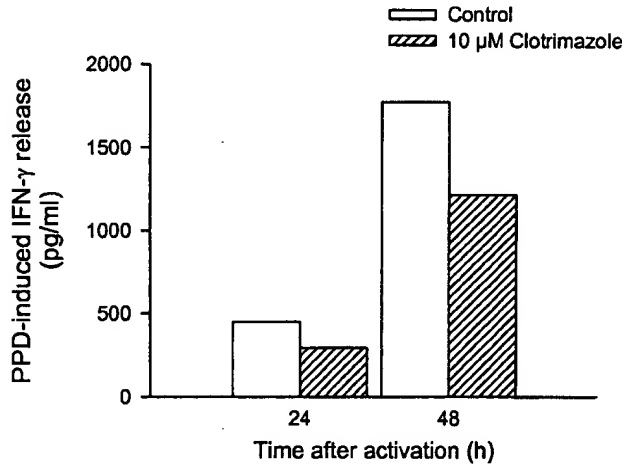

FIG. 4. Effect of clotrimazole on release of IFN-γ from activated T cells. Cells were incubated with or without PPD for the indicated periods before measurement of IFN-γ in supernatants. Similar results were obtained in two additional experiments using activation of T cells with tetanus toxin and *Candida albicans*.

IFN-γ from activated T cells by 34% in the experiment presented in Fig. 4, suggesting an inhibition of the orchestrated T cell response to antigens by the channel blocker. The average effect was an inhibition by 29% ± 5% (n = 3).

The activation of T cells via the T cell receptor is associated with activation of phospholipase C and subsequent elevation of intracellular Ca^{2+} released from intracellular stores and mobilized from the extracellular medium. The rise in $Ca^{2+}{}_i$ leads to the activation of the Ca^{2+}/calmodulin-dependent protein phosphatase 2B, also known as calcineurin, which is the target for immunosuppressive compounds such as FK506 and cyclosporin A. Concomitant inhibition of IK channels and calcineurin is expected to be additive at submaximal concentrations. Fig. 5 shows the effect of IK channel block on cyclosporin A inhibited T cell proliferation after antigen-induced T cell activation. T cells were stimulated with antigen in the presence of cyclosporin A or both cyclosporin A and clotrimazole. T cell proliferation was assayed by [^{3}H]thymidine incorporation 6 days after stimulation. The cyclosporin A-mediated inhibition of T cell proliferation is shifted leftwards by 10 μM clotrimazole, from a 50% inhibition of proliferation at approximately 25 nM cyclosporin A to half-maximal inhibition at 2.5 nM cyclosporin A. This result suggests that the antigen-induced T cell proliferation is highly sensitive to both IK channel block and inhibition of calcineurin (Fig. 5).

DISCUSSION

The data presented here demonstrate that the block of IK channels by nitrendipine, clotrimazole, and charybdotoxin leads to inhibition of the proliferative T cell response and inhibition of the release of IFN-γ from activated T cells. None of the compounds studied is selective for IK channels, but when the results are put together they support this conclusion. The inhibition of the proliferative T cell response is caused by specific interference with the pathways that induce a rise in intracellular Ca^{2+}, including those stimulated by tetanus toxin, anti-CD3, *Candida albicans*, PHA, PPD, or Con A. The increase in intracellular Ca^{2+} is recognized as an obligatory step in the cascade of signals that finally results in T cell proliferation. The early rise in Ca^{2+} is followed by a sustained increased level that results from the influx of extracellular Ca^{2+}. It was previously shown that T cell proliferation is inhibited in the absence of extracellular Ca^{2+}, and attention was focused on the role of Kv1.3 in the early cellular events following stimulation of the T cell receptor (5). Indirect results obtained by use of the selective Kv1.3 channel blockers margatoxin and noixiustoxin did lead to the conclusion that Kv1.3 dominates the K conductance of the T cell, and that inhibition of Kv1.3 is sufficient to mediate block of T cell proliferation (5). The possible involvement of IK channels in T cell activation has not been addressed previously, but there has been speculations about their playing a significant role in postactivation and secondary immune phenomena (12). Theoretically, IK channels are likely to be even more important for setting the membrane potential of T cells, since they do not close at negative membrane potentials, unlike Kv1.3, and the expression of IK channels is increased by 15- to 20-fold during T cell activation (3).

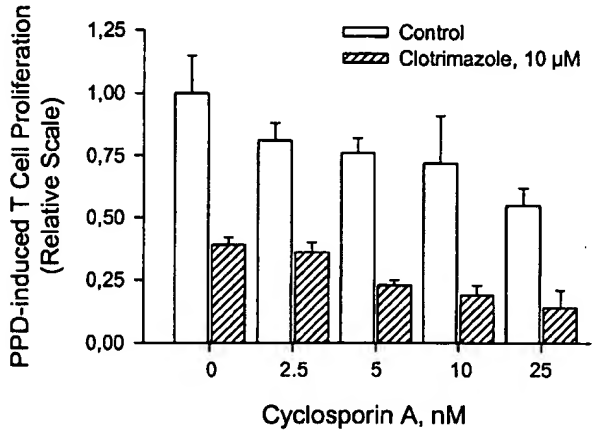

FIG. 5. Effect of clotrimazole on cyclosporin A-mediated inhibition of T cell proliferation. Cells were incubated for 5 days in culture medium with PPD in the presence of cyclosporin A alone as indicated or cyclosporin A plus clotrimazole. Clotrimazole (10 μM) was added 30 min before the addition of antigen. [^{3}H]Thymidine (1 mCi) incorporation was then measured in triplicate wells. The bars represents three independent experiments ± SE. *, $P \leq 0.05$ vs. control. Eleven other experiments using *Candida albicans*, tetanus toxin, Con A, or PHA as the antigen/mitogen challenge gave similar results.

T cell activation is accompanied not only by a rise in intracellular Ca^{2+} concentration but also by membrane hyperpolarization when stimulated by antigens or mitogens. The long-lasting hyperpolarization is the consequence of the activation of IK channels by Ca^{2+} and closely reflects the activity of intracellular Ca^{2+}. The question raised in this paper is then: how does inhibition of IK channels influence the T cell proliferation? We suggest that depolarization of T cells mediated by block of IK channels is sufficient to inhibit T cell proliferation by elimination of the inward driving force for Ca^{2+} through CRAC channels. Preliminary results from Fanger and Cahalan[¶] suggest that Th1 helper cells are subject to an increased Ca^{2+} influx after calcium depletion as compared with Th2 cells. The difference can be accounted for by a difference in IK channel expression with Th1 cells expressing significantly larger numbers of IK channels than do Th2 cells. Therefore, an additional role of IK channels in T cells might be to allow memory T cells to respond more quickly to a secondary exposure to antigens by a rapid rise in intracellular Ca^{2+} concentration caused by influx from the extracellular medium. Whether the donor variability found in this study can be accounted for by a differential expression of IK channels in the different subsets of T cells has not been investigated.

In conclusion, the present investigation suggests an additional mechanism for immune suppression. IK channels are expressed solely in peripheral tissue, and the pharmacology of IK channels is unique. These characteristics of the IK channel make it an ideal target for immune suppression. Further, it will be interesting to determine the relevance of IK channels as a target for immune suppression *in vivo*.

[¶]Fanger, C. M. & Cahalan, M. D. (1999) *Biophys. J.* **76,** A225 (abstr.).

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Minireview

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Regulation of the Calmodulin-stimulated Protein Phosphatase, Calcineurin*

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The role of protein phosphatases in the regulation of cellular processes is now well established (1, 2). Calcineurin (also called protein phosphatase 2B), a major calmodulin-binding protein in brain and the only serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin, plays a critical role in the coupling of Ca^{2+} signals to cellular responses (3–6). Its stimulation by the multifunctional protein, calmodulin, ensures the coordinated regulation of its protein phosphatase activity with the activities of the many other enzymes, including a large number of protein kinases, under Ca^{2+} and calmodulin control. Despite its special abundance in neural tissues, calcineurin is broadly distributed, and its structure is highly conserved from yeast to man (6). Its resistance to the endogenous phosphatase inhibitor 1 and inhibitor 2 and to the potent inhibitors of protein phosphatase 1 and 2A, okadaic acid, calyculin, and microcystin (1, 2) made it difficult to identify its functions until it was identified as the target of the immunosuppressive drugs, FK506 and cyclosporin A (CsA).[1] Calcineurin was thus shown to play an essential role in T cell activation (7). The demonstration that FK506 and CsA, when bound to their respective binding proteins, FKBP12 and cyclophilin A, are specific inhibitors of calcineurin provided the tools needed to reveal its many other roles in the transduction of Ca^{2+} signals (8). Its calmodulin dependence distinguishes it from two other known Ca^{2+}-regulated protein phosphatases, the insulin-sensitive pyruvate dehydrogenase phosphatase of mitochondria (9) and a family of protein phosphatases homologous to the product of the *Drosophila retinal degeneration C* (*rdgC*) gene (10–12).

Substrate Specificity and Mechanism of Action

Calcineurin has a relatively narrow substrate specificity. Phosphoproteins listed in Table I are preferentially dephosphorylated by calcineurin whereas others such as casein, synapsin 1, and calmodulin kinase II are dephosphorylated at much slower rates or not at all (5). Other potentially physiological substrates, whose kinetic characteristics have not been determined, include NO synthase, a GTPase involved in endocytosis (dynamin, previously called dephosphin), the transcription factor Elk-1, and the heat shock protein, hsp25 (13–16). The substrate specificity of calcineurin is not due only to a specific sequence but rather is determined by both primary and higher order structural features (17, 18). The phosphorylation-independent tight binding of substrates, such as described for the transcription factor NF-ATp (nuclear factor-activated T cells), may allow calcineurin to dephosphorylate proteins whose intracellular concentration is very low (19, 20). Calcineurin also dephosphorylates phosphotyrosine, but the K_{cat}, except when determined in the presence of Ni^{2+}, is 2 orders of magnitude lower than that for phosphoserine (Table I).

The synthetic peptide corresponding to residues 81–99 of the RII subunit of cAMP-dependent protein kinase (Table I) is most commonly used to measure calcineurin phosphatase activity (17). Because it is a poor substrate for protein phosphatases 1, 2A, and 2C it is well suited to quantitate the Ca^{2+}/calmodulin-dependent, metal-independent, okadaic acid-insensitive calcineurin activity in crude tissue extracts provided that the incubation time is reduced to 1–2 min to minimize Ca^{2+}-dependent calcineurin inactivation (21–24). The conveniently measurable *p*-nitrophenyl-phosphatase activity has been employed to study its catalytic mechanism and to propose a catalysis involving the protonation of the phosphoester bond by a metal-activated water molecule followed by the cleavage of the bond by a second metal-activated water molecule, without the formation of a covalent intermediate (25). This mechanism is consistent with the metal requirement for calcineurin activity (5), the identification of calcineurin as an iron-zinc enzyme (26), and the demonstration that calcineurin contains a binuclear [Fe^{3+}-Zn^{2+}] metal center (27). In the recently published crystal structures of calcineurin (28, 29), these two metal ions are modeled on the structure of the [Fe^{3+}-Zn^{2+}] kidney bean purple acid phosphatase. The high specific activity of calcineurin in crude extracts in the absence of added metals suggests that the crude enzyme has retained its natural cofactors (22). Inactivation of crude calcineurin by the superoxide anion and its protection and reactivation by ascorbate strongly suggest that reduced iron is required for activity (23).

Subunit Structure and Isoforms

Regardless of its source, calcineurin is always a heterodimer of a 58–64-kDa catalytic and calmodulin-binding subunit, calcineurin A, tightly bound (even in the presence of only nanomolar concentrations of Ca^{2+}) to a regulatory, 19-kDa Ca^{2+}-binding regulatory subunit, calcineurin B (5). This two-subunit structure, unique among the protein phosphatases, is conserved from yeast to man and is essential for activity. Also highly conserved are the amino acid sequences of the catalytic and regulatory domains of calcineurin A isoforms from different organisms (2, 6). The primary structure of the α, β, and γ isoforms of mammalian calcineurin A, products of three different genes,[2] is shown in Fig. 1. With the exception of variable N- and C-terminal tails, whose functions are not known, the three enzymes exhibit 83–89% identity over the remaining 90% of their sequence. An N-terminal polyproline motif is a conserved feature of the β isoform, whereas several additional basic residues in the C-terminal tail are responsible for the high pI (7.1) of the testis-specific γ-isoform, as opposed to pIs of 5.6 and 5.8 for the neural α and the broadly distributed β isoforms (5, 6, 30, 31).

Calcineurin B is a highly conserved protein originally identified as an "EF-hand" Ca^{2+}-binding protein on the basis of its amino acid sequence (32). Its dumbbell structure, determined by multidimensional NMR, is similar to that of calmodulin with two lobes, each composed of two adjacent Ca^{2+}-binding loops connected by a flexible helix linker (33). As predicted from its sequence, it binds 4 mol of Ca^{2+}, one with high affinity ($k_d < 10^{-7}$ M) and three with affinities in the micromolar range (34). Equally conserved from yeast to man is the myristoylation of the N-terminal glycine (35, 36). There are two mammalian isoforms of calcineurin B,[2] CNB1 originally found associated with calcineurin Aα and β and CNB2, which is expressed only in testis; only one form has been reported in fruit flies and the budding yeast (6).

Functional Domain Organization and Crystal Structure

The highly conserved multidomain structure of calcineurin A, illustrated in Fig. 1, was first revealed by limited proteolysis (37). The catalytic domain (residues 70–328 of calcineurin Aα), followed by the calcineurin B-binding domain localized by site-directed mutagenesis and binding of synthetic peptides to calcineurin B between residues 333 and 390, is resistant to proteolysis (37–43). These domains, still associated with calcineurin B, are often re-

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[1] The abbreviations used are: CsA, cyclosporin A; IP_3, inositol trisphosphate; FKBP, FK506-binding protein; NMDA, *N*-methyl-D-aspartate.

[2] The gene symbols are PPP3CAa, PPP3CAb, PPP3R1, and PPP3R2 for human calcineurin Aα, calcineurin Aβ, calcineurin B1, and calcineurin B2, respectively.

TABLE I
Substrate specificity of calcineurin

Except when indicated kinetic constants were measured in the presence of Mn^{2+} or Mg^{2+}.

Substrates	k_{cat}	k_m	Ref.
	s^{-1}	mM	
Inhibitor 1[a]	2.8	0.003	5
RII subunit	2.7	0.02	17
Neurogranin	1.8	0.013	93
Phosphorylase kinase (α subunit)[a]	1.4	0.006	5
Neuromodulin	0.1–0.5	0.015	93
Microtubule-associated proteins			
MAP-2	0.6–2.2	0.002	5
Tau factor	0.6–0.8	0.002	5
DARPP-32[a]	0.2	0.014	5
DLDVPIPGRFDRRVSVAAE[b]	2.2	0.026	94
	5.9[c]	0.023	94
DLDVPIPGRFDRRVYVAAE	<0.02		94
	0.3[c]	0.003	94
p-Nitrophenyl phosphate	26	23.0	95

[a] Values of k_{cat} measured in the absence of added metal are underestimated.

[b] Synthetic peptide routinely used for calcineurin assays. Values of 12 s^{-1} and 0.1 mM have been reported recently (62, 95). For comparison at pH 7.5 in the absence of added metal the k_{cat} value for the dephosphorylation of the peptide substrate by crude calcineurin is 40 s^{-1}.

[c] Assayed in the presence of 1 mM Ni^{2+}.

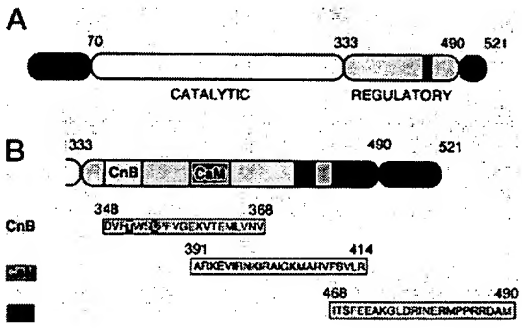

FIG. 1. **Functional domain organization of calcineurin A.** *A*, schematic representation of the three mammalian isoforms of calcineurin A. The variable regions and 10-amino acid insert, resulting from alternative splicing, in the α and β isoforms of mammalian calcineurin A are shown in *black*. *B*, extended representation of the regulatory domain; the amino acid sequences of the calcineurin B-binding helix, the calmodulin-binding domain, and the autoinhibitory peptide are *boxed*. The numbering of the amino acids is that of calcineurin Aα. Residues critical for interaction with cyclophilin and FKBP are represented by *white* on *black letters*.

ferred to as the Ca^{2+}-independent form of calcineurin. The enzymatic activity of calcineurin is repressed in the native protein, but it becomes fully active when severed by proteases from the regulatory domain (residues 390–521). The regulatory domain, readily susceptible to proteolysis in the absence of calmodulin, contains two subdomains: a calmodulin-binding and an autoinhibitory subdomain (37, 44, 45).

The crystal structures of the recombinant α isoform of human calcineurin and of its complex with FKBP12-FK506[3] (29) and that of the complex with FKBP12-FK506 (28) of the proteolytic fragment of bovine calcineurin, lacking the regulatory domain and the N-terminal 16 residues, have been determined at 2.1, 3.5, and 2.5 Å, respectively. With the exception of the N-terminal residues 1–16 and the regulatory domain of calcineurin A, missing in the bovine protein, the crystal structure of the Ca^{2+}-saturated form of the truncated bovine calcineurin shown in Fig. 2 is similar to that of the full-length recombinant protein. The catalytic domain, similar to that of protein phosphatase 1 (46), consists of a sandwich of a sheet of six β strands covered by three α helices and three β strands and a sheet of five β strands covered by an all helical structure. The two metal ions, iron and zinc, bound to residues provided by the two faces of the β sandwich, define the catalytic center. The last β sheet extends into a five-turn amphipathic α helix (residues 350–370) whose top face, completely non-polar, is covered by a 33-Å groove

[3] No changes in the structure of the recombinant calcineurin were detected upon complex formation with FKBP-FK506 (29).

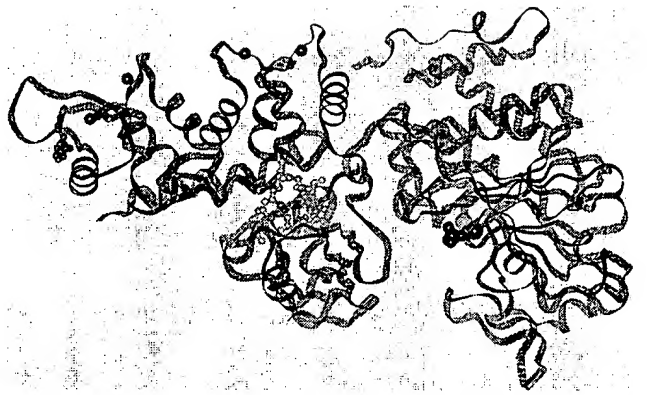

FIG. 2. **Ribbon representation of the crystal structure of truncated calcineurin complexed with FKBP12-FK506.** Calcineurin A is shown in *red* and calcineurin B in *purple* with myristic acid covalently linked to the N-terminal glycine shown in *pink*. Iron and zinc in the active site are shown as *yellow* and *green spheres* respectively, and the bound phosphate is shown in *purple*. The four Ca^{2+} in the calcineurin B sites are shown as *pink spheres*. FKBP12 is shown in *green*, and FK506 (*yellow*) is shown in ball and stick representation (Protein Data Bank code 1TCO (28)).

formed by the N- and C-terminal lobes and the C-terminal strand of calcineurin B. The contacts between the two subunits are in good agreement with the regions of calcineurin B involved in the interaction with calcineurin A (linkers between helix 1 and the Ca^{2+}-binding loop 1, Ca^{2+}-binding loops 3 and 4, the central helix linker, and the C-terminal tail) determined in solution (40, 41). Interaction of residues 14–23 of calcineurin A with the C-terminal lobe of calcineurin B may provide the additional binding energy to account for the very high affinity of calcineurin B for calcineurin A ($k_d < 10^{-13}$ M)[4] as opposed to the relatively low affinity of the calcineurin B-binding peptides of calcineurin A for calcineurin B (41, 43). In the bovine protein, myristic acid, covalently linked to the N-terminal glycine of calcineurin B, lies parallel to the hydrophobic face of the N-terminal helix of calcineurin B whereas the non-myristoylated N terminus of the recombinant protein is disordered. This perfectly conserved post-translational modification of calcineurin B, apparently not involved in membrane association, is not required for activity but may serve as a stabilizing structural element (47, 48). In the full-length protein, with the exception of two short α helices corresponding to the inhibitory domain that block the catalytic center, the regulatory domain is not visible in the electron density map (29). The disordered structure of this domain is consistent with its extreme sensitivity to proteolytic attack (37).

The polar bottom face of the calcineurin B-binding helix of calcineurin A, exposed to solvent, constitutes, together with calcineurin B, the binding domain of the FK506-FKBP12 complex. FKBP12 interacts with calcineurin B, the catalytic and the calcineurin B-binding domain of calcineurin A, whereas the interface of the calcineurin B-binding domain of calcineurin A and calcineurin B forms the binding site of FK506 (Fig. 2). Two-thirds of the surface contact between FKBP12-FK506 and calcineurin B comes from the latch region identified as the major site of interaction of calcineurin B with cyclophilin-CsA (40). This latch region formed by calcineurin B upon binding to calcineurin A may be recognized by each of the two immunosuppressive complexes, explaining their competitive binding to calcineurin (7). Thus, the conserved structural features of calcineurin are responsible for the unique ability of calcineurin to interact specifically with two classes of immunosuppressive drugs, CsA and FK506, complexed with their respective binding proteins (as reviewed in Ref. 49).

Calcium Regulation

The Ca^{2+} dependence of the phosphatase activity of calcineurin is controlled by two structurally similar but functionally different Ca^{2+}-binding proteins, calmodulin and calcineurin B (5). At less than 10^{-7} M Ca^{2+}, calcineurin B, with its high affinity site occupied, remains bound to calcineurin A, but the enzyme is inactive. Occupancy of the low affinity sites (k_d between 0.5 and 1 μM)

[4] H. Ren, X. Wang, and C. Klee, unpublished observations.

results in a small activation. The basal activity is stimulated more than 20-fold by the addition of an equimolar amount of calmodulin and is strictly the result of an increased V_{max}. Consistent with the fact that activation is the result of the Ca^{2+}-dependent binding of calmodulin to calcineurin, the concentration of Ca^{2+} needed for activation decreases with increasing concentrations of calmodulin, and the calmodulin concentration needed for activation decreases with increasing Ca^{2+} concentrations (50). The highly cooperative Ca^{2+} dependence of the calmodulin stimulation of calcineurin (Hill coefficient of 2.5–3) allows the enzyme to respond to narrow Ca^{2+} thresholds following cell stimulation. As with most calmodulin-regulated enzymes, the mechanism of calmodulin activation is believed to involve binding to the calmodulin-binding domain, resulting in a displacement of an autoinhibitory domain (5, 51). The flexible structure of the calmodulin-binding domain revealed in the crystal structure of calcineurin and the blocking of the catalytic center by the autoinhibitory domain is compatible with this mechanism, but definitive proof of this mechanism depends on the elucidation of the structure of the calcineurin-calmodulin complex.

The displacement of the inhibitory domain upon calmodulin binding can also explain the role of calmodulin in the oxidative inactivation of calcineurin. In crude tissue extracts, calcineurin exhibits a high phosphatase activity that is almost completely dependent on calmodulin and does not depend on added metals for activity but is subject to a time- and Ca^{2+}/calmodulin-dependent inactivation facilitated by small heat-stable inactivators (22). The search for factors responsible for the high phosphatase activity and instability of crude calcineurin led to the finding that, in crude extracts, calcineurin is protected against inactivation by superoxide dismutase (23). The displacement of the autoinhibitory domain upon binding of Ca^{2+}/calmodulin may expose the metal cofactors in the active site of the enzyme to the damaging effects of superoxide anion. The reversibility of calcineurin inactivation by ascorbate suggests that it is the result of the oxidation of Fe^{2+} at the active site. This protection of calcineurin activity by superoxide dismutase has also been observed in yeast cells and in hippocampal neurons after prolonged Ca^{2+} stimulation (23, 52). The modulation of calcineurin activity by the oxidation of iron provides a reversible mechanism to desensitize the enzyme and to couple Ca^{2+}-dependent protein dephosphorylation to the redox state of the cells.

The clarification of the role of calcineurin B in the Ca^{2+} regulation of calcineurin has been more elusive. Calcineurin B is required for the reconstitution of an active enzyme from its subunits separated under denaturing conditions (53) or expressed in *Escherichia coli* and SF-9 cells (40, 42, 43, 54). The irreversible inactivation and dissociation of the two subunits accompanying the complete decalcification of calcineurin indicate that Ca^{2+} binding to the high affinity site of calcineurin B plays a structural rather than a regulatory role (50). Ca^{2+} binding to the low affinity sites is apparently not only responsible for the small calmodulin-independent activation but also for calmodulin activation (50). The mechanism of calcineurin B activation has not yet been elucidated. It is not clear why the catalytic domain of calcineurin, whose structure is similar to the catalytic subunit of other protein phosphatases, is inactive or why the calmodulin-independent form of calcineurin, which still binds calcineurin B but lacks the calmodulin-binding and autoinhibitory domain, has an affinity for Ca^{2+} 10 times greater than that of the native enzyme (50).

Target Proteins

The crystal structure of the calcineurin-FKBP12-FK506 complex identified the interface between calcineurin B and calcineurin A as the binding site of the FK506-FKBP complex, providing an explanation for the requirement of both subunits for interaction first proposed on the basis of cross-linking experiments (55) and confirmed by affinity labeling and site-directed mutagenesis (38–40, 56–58). It also confirmed the identification of the calcineurin-binding domain of FK506 predicted on the basis of the structural and functional differences between FK506 and rapamycin and the preferential binding of the FKBP12 among FK506-binding proteins (49). Residues of FKBP12 and cyclophilin A involved in interaction with calcineurin are distinct from the isomerase catalytic and drug-binding sites in agreement with the lack of correlation between isomerase activity and calcineurin inhibition (59–62). The apparent competitive binding of two structurally different drug complexes, FKBP-FK506 and cyclophilin-CsA, to the same site on calcineurin remained a puzzle until the isolation of calcineurin A mutants that are resistant to either FK506 or CsA, indicating that the interaction sites are overlapping but not identical (57, 58).

The conservation from yeast to man of the drug-binding domains of calcineurin raises the possibility that these domains interact with natural ligands. Although no small endogenous analogs of FK506 and CsA have been detected, the disruption of the FKBP12-mediated anchoring of calcineurin to the ryanodine and IP_3 receptors by FK506 suggests that these receptors may be such analogs (63, 64). The FKBP-mediated targeting of calcineurin to the receptors may ensure a rapid modulation of Ca^{2+} release from internal stores by protein dephosphorylation (65, 66). Another potentially important partner of calcineurin is AKAP79 (A-kinase anchoring protein), a member of the family of proteins whose function is to bring kinases or phosphatases close to their substrates (67). Like the IP_3 and ryanodine receptors, AKAP79 requires neither FK506 nor Ca^{2+} to bind calcineurin. The tight, phosphorylation-independent binding of the transcription factor NF-AT to calcineurin may be another example of calcineurin targeting mediated by the substrate itself (20). It remains to be shown if the binding sites for these target proteins overlap with the drug-binding site.

Calcineurin Functions

The complex regulation of calcineurin is expected from an enzyme, which has now been shown to be a major player in the regulation of cellular processes. None is better understood than the Ca^{2+}-dependent, calcineurin-mediated regulation of transcription of the T cell growth factor, interleukin-2 (49, 68–70). The translocation of the transcription factor, NF-ATp, in response to an increase of intracellular Ca^{2+} induced by the occupancy of the T cell receptor, is dependent upon its dephosphorylation by calcineurin. It was the first example of the transduction of a signal at the plasma membrane to the nucleus (7, 19). A prolonged Ca^{2+} signal and the cotranslocation of calcineurin and NF-ATp to the nucleus ensure the sustained activation of gene expression, which is reversed by the glycogen synthase kinase, GSK3, following a decrease of intracellular Ca^{2+} (71–73). The involvement of calcineurin in the regulation of the expression of an array of growth factors, κ3, TNFα, NFκB, NF(P), and TGFβ, is reviewed in Refs. 68–70 and 74.

As it does in T cells, calcineurin plays an important role in the regulation of gene expression in response to Ca^{2+} signals in yeast (70, 75–79). Two major sites of action of calcineurin in this organism are the pheromone response pathway (36) and the adaptation to high salt stress (75). The induction of genes involved in these two pathways has now been shown to be regulated differentially by the Ca^{2+}-dependent and FK506-sensitive interaction of a single transcription factor (Tcn1p also named Crz1p) with calcineurin (76, 77). Other processes under calcineurin control include Ca^{2+} sequestration, cytokinesis, sporulation, and mating (78–80).

Identifying the sites of action of calcineurin in striatal and hippocampal neurons, which are particularly rich in calcineurin, continues to be a major challenge. The dephosphorylation of DARPP-32 by calcineurin in striatal neurons was the first evidence for a protein phosphatase cascade involving calcineurin responsible for the opposite effects of glutamate and cAMP on neuronal excitability (81). In hippocampal neurons activation of calcineurin not only results in inhibition of the release of the neurotransmitters, glutamate and γ-aminobutyric acid (82, 83), but is also involved in the desensitization of the postsynaptic NMDA receptor-coupled Ca^{2+} channels (84). The complex regulation of the function of the NMDA receptors may be the basis for the proposed role of calcineurin in long term potentiation and depression and long term memory (85, 86). Calcineurin-mediated activation of nitric oxide synthase has also been invoked to explain glutamate neurotoxicity (87).

A role for calcineurin has also been proposed in apoptosis (88) and in the redistribution of integrins required for the migration of neutrophils on vibronectin in response to Ca^{2+} transients (89, 90). The inhibition of the calcineurin-mediated regulation of the Na^+,K^+-ATPase by the immunosuppressive drugs in the kidney (91) may be responsible for their nephrotoxicity, whereas in cerebellar neurons, the calcineurin activation of the Na^+,K^+-ATPase is

required to prevent neurotoxicity because of excessive Na^+ entry induced by glutamate binding to NMDA receptors (92). Regulation of two intracellular Ca^{2+} channels (the ryanodine receptor involved in excitation-contraction coupling in skeletal muscle and other excitable cells and the IP_3 receptor involved in Ca^{2+} release by hormones and neurotransmitters) can potentially affect all cellular processes under Ca^{2+} control.

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